

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

THIS PAGE BLANK (USPTO)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

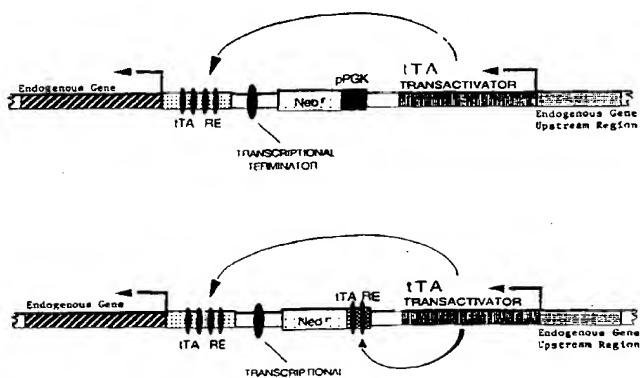
| | | | |
|--|--|---|---|
| (51) International Patent Classification 5 : C12N 15/00, 15/12, 15/62, 15/85, 15/90, 5/10, 1/19, 1/15, A01K 67/027 | | A2 | (11) International Publication Number: WO 94/29442 (43) International Publication Date: 22 December 1994 (22.12.94) |
| (21) International Application Number: PCT/US94/06734 | | (74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US). | |
| (22) International Filing Date: 14 June 1994 (14.06.94) | | (81) Designated States: AU, CA, JP, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). | |
| (30) Priority Data: 08/076,327 14 June 1993 (14.06.93) US | | Published <i>Without international search report and to be republished upon receipt of that report.</i> | |
| (60) Parent Application or Grant (63) Related by Continuation US Filed on | | Not furnished (CIP) Not furnished | |
| (71) Applicant (for all designated States except US): BASF AKTIENGESELLSCHAFT [DE/DE]; D-67056 Ludwigshafen (DE). | | | |
| (72) Inventors; and (75) Inventors/Applicants (for US only): BUJARD, Hermann [DE/DE]; Remler Strasse 9, D-69120 Heidelberg (DE). GOSSEN, Manfred [DE/DE]; Im Neuenheimer Feld 282, D-69120 Heidelberg (DE). SALFELD, Jochen, G. [DE/US]; 177 Old Westboro Road, North Grafton, MA 01536 (US). VOSS, Jeffrey, W. [US/US]; Apartment 10, 35 Georgetown Road, Framingham, MA 01701 (US). | | | |

(54) Title: TIGHT CONTROL OF GENE EXPRESSION IN EUKARYOTIC CELLS BY TETRACYCLINE-RESPONSIVE PROMOTERS

(57) Abstract

Transgenic animals carrying two transgenes, the first coding for a transactivator fusion protein comprising a tet repressor and a polypeptide which directly or indirectly activates in eukaryotic cells, and the second comprising a gene operably linked to a minimal promoter operably linked to at least one tet operator sequence, are disclosed. Isolated DNA molecules (e.g., targeting vectors) for integrating a polynucleotide sequence encoding a transactivator of the invention at a predetermined location within a second target DNA molecule by homologous recombination are also disclosed. Transgenic animals having the DNA molecules of the invention integrated at a predetermined location in a chromosome by homologous recombination are also encompassed by the invention. Methods to regulate the expression of a tet operator linked gene of interest by administering tetracycline or a tetracycline analogue to an animal of the invention are also disclosed. The regulatory system of the invention allows for conditional inactivation or modulation of expression of a gene of interest in a host cell or animal.

Conditional Knock-Out Strategy 3



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AT | Austria | GB | United Kingdom | MR | Mauritania |
| AU | Australia | GE | Georgia | MW | Malawi |
| BB | Barbados | GN | Guinea | NE | Niger |
| BE | Belgium | GR | Greece | NL | Netherlands |
| BF | Burkina Faso | HU | Hungary | NO | Norway |
| BG | Bulgaria | IE | Ireland | NZ | New Zealand |
| BJ | Benin | IT | Italy | PL | Poland |
| BR | Brazil | JP | Japan | PT | Portugal |
| BY | Belarus | KE | Kenya | RO | Romania |
| CA | Canada | KG | Kyrgyzstan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SI | Slovenia |
| CI | Côte d'Ivoire | LJ | Liechtenstein | SK | Slovakia |
| CM | Cameroon | LK | Sri Lanka | SN | Senegal |
| CN | China | LU | Luxembourg | TD | Chad |
| CS | Czechoslovakia | LV | Latvia | TG | Togo |
| CZ | Czech Republic | MC | Monaco | TJ | Tajikistan |
| DE | Germany | MD | Republic of Moldova | TT | Trinidad and Tobago |
| DK | Denmark | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | US | United States of America |
| FI | Finland | MN | Mongolia | UZ | Uzbekistan |
| FR | France | | | VN | Viet Nam |
| GA | Gabon | | | | |

Tight Control of Gene Expression in Eucaryotic Cells
by Tetracycline-Responsive Promoters

5

Background of the Invention

The study of gene function in complex genetic environments such as eucaryotic cells would greatly profit from systems that would allow stringent control of the expression of individual genes. Ideally, such systems would not only mediate an "on/off" status for gene expression but would also permit limited expression at a defined level.

Attempts to control gene activity by various inducible eucaryotic promoters responsive to, for example, heavy metal ions (Mayo et al., Cell 29:99-108 (1982); Brinster et al., Nature (London) 296:39-42 (1982); Searle et al., Nouer, L., CRC Boca Raton, FL (1991), pp. 167-220), or hormones (Lee et al., Nature (London) 294:228-232 (1981); Hynes et al., Proc. Natl. Acad. Sci. USA 78:2038-2042 (1981); Klock et al., Nature (London) 329:734-736 (1987); Israel & Kaufman, Nucleic Acids Res. 17:2589-2604 (1989)) have generally suffered from leakiness of the inactive state (e.g., the metallothionein promoter (Mayo et al., Cell 29:99-108 (1982)) or from pleiotropic effects caused by the inducing principles themselves, such as elevated temperature or glucocorticoid hormone action (Lee et al., Proc. Natl. Acad. Sci, USA 85:1204-1208 (1988)).

In search of regulatory systems that do not rely on endogenous control elements, several groups have demonstrated that the lac repressor/operator inducer system of Escherichia coli functions in eucaryotic cells. Three approaches have been described: (i) prevention of transcription initiation by properly placed lac operators at promoter sites (Hu & Davidson, Cell 48:555-566 (1987); Brown et al., Cell 49:603-612 (1987); Figge et al., Cell 52:713-722 (1988); Fuerst et al., Proc. Natl. Acad. Sci. USA 86:2549-2553 (1989); Deutschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5405 (1989)), (ii) blockage of transcribing RNA polymerase II during elongation by a lac repressor/operator complex (lac R/O; Deutschle et al., Science 248:480-483 (1990)), and (iii) activation of a promoter responsive to a fusion between lacR and the activating domain of virion protein 16 (VP16) of herpes simplex virus (HSV) (Labow et al., Mol. Cell. Biol. 10:3343-3356 (1990); Baim et al., Proc. Natl. Acad. Sci. USA 88:5072-5076 (1991)).

At present, however, the utility of the lacR/O-based systems in eucaryotic cells is limited since the inducer isopropyl- β -D-thiogalactopyranoside (IPTG), despite its rapid uptake and intracellular stability (Wyborski & Short, Nucleic Acids Res. 19:4647-4653), acts rather slowly and inefficiently, resulting in only moderate induction. Nevertheless, an interesting conditional mutant of a lacR-VP16 fusion has been described (Baim et al., Proc. Natl. Acad. Sci. USA 88:5072-5076 (1991)). It activates a minimal promoter ~ 1000-fold at elevated temperatures in the presence of IPTG. The temperature dependence and the inherent

IPTG-related problems, however, may also limit this approach.

Summary of the Invention

This invention features a system for regulating expression of eucaryotic genes using components of the Tet repressor/operator/ inducer system of prokaryotes. In the system of the invention, transcription of a nucleotide sequence operably linked to at least one *tet* operator sequence is stimulated by a tetracycline (Tc)-controllable transcriptional activator fusion protein (referred to herein as tTA). The tTA is comprised of two polypeptides. The first polypeptide is a Tet repressor (TetR; e.g., a Tn10-derived TetR), which binds to *tet* operator sequences in the absence but not the presence of Tc. The second polypeptide directly or indirectly activates transcription in eucaryotic cells. For example, the second polypeptide can be a transcriptional activation domain from herpes simplex virus virion protein 16 or another transcriptional activating domain, e.g. acidic, proline-rich, serine/threonine-rich, glutamine-rich. Alternatively, the second polypeptide can be a domain (e.g., a dimerization domain) which recruits a transcriptional activator (e.g., an endogenous transcriptional activator) to interact with the tTA fusion protein by a protein-protein interaction (e.g., a non-covalent interaction). In the absence of Tc or a Tc analogue, transcription of a gene operably linked to a tTA-responsive promoter (typically comprising at least one *tet* operator sequence and a minimal promoter) is stimulated by a tTA of the invention, whereas in the presence of Tc or a Tc analogue, transcription of the gene linked to the tTA-responsive promoter is not stimulated by the tTA.

As described herein, this system functions effectively in transgenic animals. Accordingly, the invention provides a tetracycline-controllable regulatory system for modulating gene expression in transgenic animals. Additionally, the invention provides targeting vectors for homologous recombination that enable the components of the regulatory system to be integrated at a predetermined location in the genome of a host cell or animal. This embodiment of the invention is able to solve a longstanding problem in the field generally described as gene targeting or gene knock out. Constitutive disruption of certain genes has been found to produce lethal mutations resulting in death of homozygous embryos, e.g., as described for the knock out of the RB gene (Jacks, T. et al. (1992) Nature 359:295-300). This problem precludes the development of "knock-out" animals for many genes of interest. The regulatory system of the invention can be applied to overcome this problem. DNA encoding a tTA of the invention can be integrated within a gene of interest such that expression of the tTA is controlled by the endogenous regulatory elements of the gene of interest (e.g., the tTA is expressed spatially and temporally in a manner similar to the gene of interest). The gene of interest is then operably linked to at least one *tet* operator sequence (either at its endogenous site by homologous recombination or a second copy of the gene of interest, linked to *tet* operator(s), can be integrated at another site). Expression of the *tet*-operator linked gene is thus placed under the control of the tTA, whose pattern of

expression mimics that of the gene of interest. In the absence of Tc, expression of the tet operator-linked gene of interest is stimulated by the tTA and the animal develops like a nonmutated wildtype animal. Then, at a particular stage of development, expression of the gene of interest can be switched off by raising the level of Tc (or a Tc analogue) in the circulation and the tissues of the animal by feeding or injecting Tc (or a Tc analogue) to the animal, thereby inhibiting the activity of the tTA and transcription of the gene of interest. This method is generally referred to herein as a "conditional knockout".

Accordingly, one aspect of the invention relates to targeting vectors for homologous recombination. In one embodiment, the invention provides an isolated DNA molecule for integrating a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) at a predetermined location in a second target DNA molecule. In this DNA molecule, a polynucleotide sequence encoding a tTA is flanked at 5' and 3' ends by additional polynucleotide sequences of sufficient length for homologous recombination between the DNA molecule and the second target DNA molecule at a predetermined location. Typically, the target DNA molecule into which the tTA-coding sequences are integrated is a gene of interest, or regulatory region thereof, in a eucaryotic chromosome in a host cell. For example, tTA-coding sequences can be inserted into a gene within a yeast, fungal, insect or mammalian cell. Additionally, tTA-coding sequences can be inserted into a viral gene present within a host cell, e.g. a baculovirus gene present in insect host cell. In a preferred embodiment, integration of the tTA-encoding sequences into a predetermined location in a gene of interest (by homologous recombination) places the tTA-coding sequences under the control of regulatory elements of the gene of interest (e.g., 5' flanking regulatory elements), such that the tTA is expressed in a spatial and temporal manner similar to the gene of interest.

In another embodiment of the targeting vector for homologous recombination, the isolated DNA molecule permits integration of a polynucleotide sequence encoding both a tTA and a tTA-responsive promoter within a predetermined gene of interest in a second target DNA molecule (a "single hit vector", schematically illustrated in Figure 13A-B). This molecule includes: 1) a first polynucleotide sequence comprising a 5' flanking regulatory region of the gene of interest, operably linked to 2) a second polynucleotide sequence encoding a tTA; and 3) a third polynucleotide sequence comprising a tTA-responsive promoter, operably linked to: 4) a fourth polynucleotide sequence comprising at least a portion of a coding region of the gene of interest. The first and fourth polynucleotide sequences are of sufficient length for homologous recombination between the DNA molecule and the gene of interest such that expression of the tTA is controlled by 5' regulatory elements of the gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter (i.e., upon binding of the tTA to the tTA-responsive promoter, expression of the gene of interest is stimulated). This targeting vector can also include a polynucleotide sequence encoding a selectable marker operably linked to a regulatory sequence. Typically, the selectable marker expression unit is located between the tTA-

encoding sequence (i.e., the second polynucleotide sequence described above) and the tTA-responsive promoter (i.e., the third polynucleotide sequence described above). Additionally or alternatively, this targeting vector can also include a sequence, typically located upstream (i.e., 5') of the tTA-responsive promoter (e.g., between the selectable marker expression unit 5 and the tTA responsive promoter) which terminates transcription or otherwise insulated downstream elements from the effects of upstream regulatory elements. The tTA-responsive promoter typically includes a minimal promoter operably linked to at least one tet operator sequence. The minimal promoter is derived, for example, from a cytomegalovirus immediate early gene promoter or a herpes simplex virus thymidine kinase gene promoter.

Another aspect of the invention relates to eucaryotic host cells containing a DNA molecule encoding a tTA integrated at a predetermined location in a second target DNA molecule (e.g., a gene of interest) in the host cell. Such a eucaryotic host cell can be created by introducing a targeting vector of the invention into a population of cells under conditions suitable for homologous recombination between the DNA encoding the tTA and the second target DNA molecule and selecting a cell in which the DNA encoding the tTA has integrated at a predetermined location within the second target DNA molecule. The host cell can be a mammalian cell (e.g., a human cell). Alternatively, the host cell can be a yeast, fungal or insect cell (e.g., the tTA-encoding DNA can be integrated into a baculovirus gene within an insect cell). A preferred host cell type for homologous recombination is an embryonic stem 10 cell, which can then be used to create a non-human animal carrying tTA-coding sequences integrated at a predetermined location in a chromosome of the animal. A host cell can further contain a gene of interest operably linked to a tTA-responsive transcriptional promoter. The gene of interest operably linked to the tTA-responsive promoter can be integrated into DNA of the host cell either randomly (e.g., by introduction of an exogenous gene) or at a 15 predetermined location (e.g., by targeting an endogenous gene for homologous recombination). The gene linked to the tTA-responsive promoter can be introduced into the host cell independently from the DNA encoding the tTA, or alternatively, a "single hit" targeting vector of the invention can be used to integrate both tTA-coding sequences and a tTA-responsive promoter into a predetermined location in DNA of the host cell. Expression 20 of a gene of interest operably linked to a tTA-responsive promoter in a host cell of the invention can be inhibited by contacting the cell with tetracycline or a tetracycline analogue.

Another aspect of the invention relates to non-human transgenic animals having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) of the invention or having a transgene encoding a gene of interest 25 operably linked to a tTA-responsive promoter. Double transgenic animals having both transgenes (i.e., a tTA-coding transgene and a gene of interest linked to a tTA-responsive promoter) are also encompassed by the invention. In one embodiment, the transgenic animal is a mouse. In other embodiments, the transgenic animal is a cow, a goat, a sheep and a pig. Transgenic animals of the invention can be made, for example, by introducing a DNA 30

molecule encoding the tTA or the gene of interest operably linked to a tTA responsive promoter into a fertilized oocyte, implanting the fertilized oocyte in a pseudopregnant foster mother, and allowing the fertilized oocyte to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal. Double transgenic animals can be
5 created by appropriate mating of single transgenic animals. Expression of a gene of interest operably linked to a tTA responsive promoter in a double transgenic animal of the invention can be inhibited by administering tetracycline or a tetracycline analogue to the animal.

Another aspect of the invention relates to non-human transgenic animals having a transgene encoding a tTA of the invention, wherein the transgene is integrated by
10 homologous recombination at a predetermined location within a chromosome within cells of the animal (also referred to herein as a homologous recombinant animal). The homologous recombinant animal can also have a second transgene encoding a gene of interest operably linked to a tTA-responsive promoter. The second transgene can be introduced randomly or, alternatively, at a predetermined location within a chromosome (e.g., by homologous
15 recombination. For example, a single hit vector of the invention can be used to create a homologous recombinant animal in which expression of the tTA is controlled by 5' regulatory elements of a gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter (such that in the absence of Tc, expression of the gene is stimulated by tTA binding to the tTA responsive promoter).

20 A non-human transgenic animal of the invention having tTA-coding sequences integrated at a predetermined location within chromosomal DNA of cells of the animal can be created by introducing a targeting vector of the invention into a population of embryonic stem cells under conditions suitable for homologous recombination between the DNA encoding the tTA and chromosomal DNA within the cell, selecting an embryonic stem cell in which
25 DNA encoding the tTA has integrated at a predetermined location within the chromosomal DNA of the cell, implanting the embryonic stem cell into a blastocyst, implanting the blastocyst into a pseudopregnant foster mother and allowing the blastocyst to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal.

Another aspect of the invention relates to a process for producing and isolating a gene
30 product (e.g., protein) encoded by a gene of interest operably linked to a tTA-responsive transcriptional promoter in a host cell of the invention carrying tTA-coding sequences. In the process, a host cell is first grown in a culture medium in the presence of tetracycline or a tetracycline analogue (under these conditions, expression of the gene of interest is not stimulated). Next, the concentration of tetracycline or the tetracycline analogue in the culture
35 medium is reduced to stimulate transcription of the gene of interest. The cells are further cultured until a desired amount of the gene product encoded by the gene of interest is produced by the cells. Finally, the gene product is isolated from harvested cells or from the culture medium. Preferred cells for use in the process include yeast or fungal cells.

Kits containing the components of the regulatory system of the invention described

herein are also within the scope of the invention.

Various additional features, components and aspects of this invention are described in further detail below.

5 **Brief Description of the Figures**

Fig. 1 (panels a and b). Schematic representation of tetR-VP16 fusion proteins (tTAs), encoded by plasmids pUHD15-1 and pUHD151-1, and a tTA-dependent transcription unit, encoded by plasmid PUHC13-3.

Panel a: Diagrammatic representation of two tTA proteins. In both fusion proteins, 10 tTA and tTAs, the original 207-amino-acid sequence of tetR is conserved. Two versions of VP 16 sequences encoding the activation domain were fused in frame to the 3' end of the tetR gene, resulting in tTA and tTAs. The bold letters indicate the original amino acids at the N terminal end, the junction, and the C-terminal end of the fusion proteins; the other letters designate amino acids introduced due to sequence constraints of the particular system. The 15 numbers delineate amino acid positions within tetR (Hillegaart and Wissman in Protein-Nucleic Acid Interaction, Topics in Molecular and Structural Biology, Saenger and Heinemann (eds.), Vol 10, pp. 143-162 (1989)) or VP16 (Trezenberg et al., Genes Dev. 2:718-729 (1988)), respectively.

Panel b: The tTA-dependent transcriptional unit consists of the simian virus 40 (SV40) poly(A) site (An), the luciferase gene (luc), the PhCMV*-1 or PhCMV*-2. The two 20 promoters encompass the sequence between +75 and -53 of the PhCMV*-2 with one base-pair exchange at -31, which creates a Stu I cleavage site. The Xho I site introduced at -53 by PCR was utilized to insert the heptamerized tetO sequence. This heptameric sequence is flanked at one side by an 18-nucleotide polylinker, which allows the insertion of the 25 operators in both orientations as Sal I/Xhol fragments. The position of the central G/C base pair of the promoter proximal operator to position +1 is -95 for PhCMV*-1 (upper construct) and -76 for PhCMV*-2 (lower construct). The plasmids that contain the four constructs are indicated on the far right.

Fig. 2 (panels a and b). Western blots showing the identification and characterization 30 of tTA produced in HeLa cells. HeLa cells grown to 40% confluence were transiently transfected with pUHD15-1 by the calcium phosphate method. Nuclear and cytoplasmic extracts were prepared after 36 hr.

Panel a: Western blot analysis of electrophoretically separated extracts (6% acrylamide/0.1% SDS gels) with tetR-specific antibodies reveals a protein of about 37 kDa 35 (tTA) in cytoplasmic (C) and nuclear (N) extracts in pUHD15-1 transfected cells (+) that is not present in mock-transfected cells (-).

Panel b: Mobility change of tetO DNA by tTA binding from HeLa cell nuclear extracts. Radioactively labeled tetO DNA was mixed with extracts from mock-transfected (lanes 2 and 3) and pUHD15-1-transfected (lanes 4 and 5) HeLa cells in the absence (lanes 2

and 4) and presence (lanes 3 and 5) of 1 µg of tetracycline per ml (added 2 min prior to the addition of the operator). Lane 1 contains labeled operator DNA only.

Fig. 3 (panels a and b). Graphs showing the dependence of tTA function on tetracycline.

5 Panel a: Dependence of luciferase (luc.) activity on tetracycline concentration. HeLa cell clones X1 (dashed line) and T12 previously grown in tetracycline-free medium were seeded with a density of 5000 cells per 35mm dish and incubated at the tetracycline concentrations indicated. After reaching ~ 90% confluence, cells were harvested and assayed for luciferase activity. Data given are the means ± SD of three independent experiments.

10 Panel b: Kinetics of tetracycline action. X1 cells were grown in 100mm dishes to ~ 80% confluence in the absence or presence (0.1 µg/ml) of tetracycline. At time 0, cells were washed with phosphate-buffered saline and split into smaller culture dishes (1/20th of the initial cultures per 35mm dish). Half of the cultures remained in tetracycline-free medium (■) and the other half were incubated in the presence of tetracycline (1 µg/ml; □). The X1 15 culture grown in tetracycline-containing medium was split in the same manner; one half was further incubated in the presence of tetracycline (●), whereas the other half was transferred to tetracycline-free medium (○). At the times indicated, aliquots were harvested and examined for luciferase activity. The slight increase in luciferase activity monitored at 4 hr in the culture containing tetracycline (●) is reproducible and reflects luciferase induction during the washing step.

20 Fig. 4. [SEQ ID NO: 1] The polynucleotide sequence coding for tTA transactivator.

Fig. 5. [SEQ ID NO: 3] The polynucleotide sequence coding for tTAS transactivator.

25 Fig. 6. [SEQ ID NO: 5] The polynucleotide sequence of PhCMV*-1. The nucleotide sequence shown encompasses the tet operator sequences (*italics*) and the hCMV minimal promoter, of which position -53, the TATA box and position +75 (relative to the transcription start site) are underlined.

30 Fig. 7. [SEQ ID NO: 6] The polynucleotide sequence of PhCMV*-2. The nucleotide sequence shown encompasses the tet operator sequences (*italics*) and the hCMV minimal promoter, of which position -53, the TATA box and position +75 (relative to the transcription start site) are underlined .

35 Fig. 8. [SEQ ID NO: 7] The polynucleotide sequence of PTK*-1. The nucleotide sequence shown encompasses the tet operator sequences (*italics*) and the HSV-Tk minimal promoter, of which position -81, the TATA box and position +7 (relative to the transcription start site) are underlined .

Fig. 9A-9C. [SEQ ID NO: 8] The polynucleotide sequence of the cDNA coding for the rabbit progesterone receptor under control of PhCMV*-1

Fig. 10A-B. [SEQ ID NO: 9] The polynucleotide sequence of the cDNA coding for the rabbit progesterone receptor under control of PhCMV*-1

Fig. 11. A schematic representation of Conditional Knock Out Strategy 1 in which

"E.G. 5'" represents flanking nucleotide sequence from 5' of the coding sequence for an Endogenous Gene; "E.G. 3'" represents flanking nucleotide sequence from 3' of the coding sequence for an Endogenous Gene; and "tTARE" represents a tTA responsive element inserted just upstream of a copy of the endogenous gene of interest. (In other embodiments 5 the gene linked to the tTA is a heterologous gene.)

Fig. 12. A schematic representation of Conditional Knock Out Strategy 2 in which "tTARE" is a tTA responsive promoter element: "E.G.". is an endogenous gene; "E.G. 5'" represents flanking nucleotide sequence from 5' of the coding sequence for an Endogenous Gene; "E.G. 3'" represents flanking nucleotide sequence from 3' of the coding sequence for 10 an Endogenous Gene; and "TK" is a thymidine kinase gene.

Fig. 13.A-B A schematic representation of Conditional Knock Out Strategy 3 depicting vector designs in which abbreviations are as defined above, Neo^r is a neomycin resistance gene and pPGK is phosphoglycerate kinase sequence.

Fig. 14. A graphic representation of the doxycycline dependent luciferase activity in 15 double transgenic mice carrying PhCMV-tTA and PhCMV*-1-luc transgenes. Light bars show tTA-activated luciferase levels in different tissues from 2 individual mice. Dark bars show luciferase levels in different tissues from 2 individual mice that received doxycycline in the drinking water (200 mg/ml, 5 % sucrose) for 7 days. Spotted bars (controls) represent the 20 average luciferase background activity from 5 individuals from line L7, carrying only the PhCMV*-1-luc transgene.

Detailed Description of the Invention

Definitions

25 The description that follows makes use of a number of terms used in recombinant DNA technology. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cloning Vector. A plasmid or phage DNA or other DNA sequence which is able to 30 replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells 35 transformed with the cloning vector.

Expression Vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either

constitutive or inducible.

Eucaryotic Host Cell. According to the invention, a eucaryotic host cell may be any such cell which include, but are not limited to, yeast cells, plant cells, fungal cells, insect cells, e.g. Schneider and sF9 cells, mammalian cells, e.g. HeLa cells (human), NIH3T3 (murine), RK13 (rabbit) cells, embryonic stem cell lines, e.g. D3 and J1, and cell types such as hematopoietic stem cells, myoblasts, hepatocytes, lymphocytes, airway epithelium and skin epithelium.

Recombinant Eucaryotic Host. According to the invention, a recombinant eucaryotic host may be any eucaryotic cell which contains the polynucleotide molecules of the present invention on an expression vector or cloning vector. This term is also meant to include those eucaryotic cells that have been genetically engineered to contain the desired polynucleotide molecules in the chromosome, genome or episome of that organism. Thus, the recombinant eucaryotic host cells are capable of stably or transiently expressing the proteins.

Recombinant vector. Any cloning vector or expression vector which contains the polynucleotide molecules of the invention.

Host. Any prokaryotic or eucaryotic cell that is the recipient of a replicable vector. A "host" as the term is used herein, also includes prokaryotic or eucaryotic cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, see Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

Promoter. A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Minimal Promoter. A partial promotor sequence which defines the transcription start site but which by itself is not capable, if at all, of initiating transcription efficiently. The activity of such minimal promoters depend on the binding of activators such as a tetracycline-controlled transactivator to operably linked binding sites.

Gene. A DNA sequence that contains information needed for expressing a polypeptide or protein.

Structural Gene. A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Polynucleotide molecules. A polynucleotide molecule may be a polydeoxyribonucleic acid molecule (DNA) or a polyribonucleic acid molecule (RNA).

Complementary DNA (cDNA). A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Expression. "Expression" is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

5 Fragment. A "fragment" of a molecule is meant to refer to any polypeptide subset of that molecule.

Tet repressor. A "tet repressor" refers to a prokaryotic protein which binds to a tet operator sequence in the absence but not the presence of tetracycline. The term "tet repressor" is intended to include repressors of different class types, e.g., class A, B, C, D or E tet repressors.

10 Tetracycline Analogue. A "tetracycline analogue" is any one of a number of compounds that are closely related to tetracycline (Tc) and which bind to the tet repressor with a K_a of at least about 10^6 M^{-1} . Preferably, the tetracycline analogue binds with an affinity of about 10^9 M^{-1} or greater, e.g. 10^9 M^{-1} . Examples of such tetracycline analogues include, but are not limited to those disclosed by Hlavka and Boothe, "The Tetracyclines," in 15 Handbook of Experimental Pharmacology 78, R.K.. Blackwood et al. (eds.), SpringerVerlag, Berlin-New York, 1985; L.A. Mitscher "The Chemistry of the Tetracycline Antibiotics, Medicinal Research 9, Dekker, New York, 1978; Noyee Development Corporation, "Tetracycline Manufacturing Processes," Chemical Process Reviews, Park Ridge, N.J., 2 volumes, 1969; R.C. Evans, "The Technology of the Tetracyclines," Biochemical Reference 20 Series 1, Quadrangle Press, New York, 1968; and H.F. Dowling, "Tetracycline," Antibiotics Monographs, no. 3, Medical Encyclopedia, New York, 1955; the contents of each of which are fully incorporated by reference herein. Examples of tetracycline analogues include anhydrotetracycline, doxycycline, chlorotetracycline, epoxytetracycline, and the like. Certain Tc analogues, such as anhydrotetracycline and epoxytetracycline, have reduced 25 antibiotic activity compared to Tc.

Transgenic Animal. A transgenic animal is an animal having cells that contain a transgene, wherein the transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic, stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome 30 of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. Non-human animals into which transgenes can be introduced by techniques known in the art include mice, goats, sheep, pigs, cows and other domestic farm animals.

A transgenic animal can be created, for example, by introducing a nucleic acid 35 encoding a protein of interest (typically linked to appropriate regulatory elements, such as a constitutive or tissue-specific enhancer) into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. Methods for generating transgenic

animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009 and Hogan, B. et al., (1986) *A Laboratory Manual*, Cold Spring Harbor, New York, Cold Spring Harbor Laboratory. A transgenic founder animal can be used to breed additional animals carrying the transgene. A transgenic animal carrying one transgene can further be bred to another transgenic animal carrying a second transgenes to create a so-called "double transgenic" animal carrying two transgenes.

10 Homologous Recombinant Animal. The term "homologous recombinant animal" as used herein is intended to describe an animal containing a gene which has been modified by homologous recombination between the gene and a DNA molecule introduced into an embryonic cell of the animal, or ancestor thereof. Thus, a homologous recombinant animal is a type of transgenic animal in which the transgene is introduced into a predetermined chromosomal location in the genome of the animal by homologous recombination.

15 To create such a homologous recombinant animal, a vector is prepared which contains DNA of interest (e.g., encoding a tTA of the invention) flanked at its 5' and 3' ends by additional nucleic acid of a eucaryotic gene of interest at which homologous recombination is to occur. The flanking nucleic acid is of sufficient length for successful homologous recombination with the eucaryotic gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K.R. and Capecchi, M. R. 20 (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in 25 *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harbouring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by so-called "germline 30 transmission". Animals carrying the recombined gene can be bred to homozygosity and/or bred with other animals carrying other transgenes.

Recombinant expression of proteins is commonly done using constitutive promoters like human CMV (Boshart, M. et al. 1985, *Cell* Vol. 41, 521-530) or the adenovirus major 35 late promoter or SV40 early promoter as described below (see also, e.g., Kaufman, R.J. 1990 *Meth Enzymol.* Vol. 185: 537-566 and Benoist C. et al. (1981) *Nature* Vol.290:304 ff). However, in the case of proteins such as certain proteases, cytotoxic or cytostatic proteins that interfere with the cell membranes or proteins like certain receptors, whose normal biological function triggers a response to the host cell environment (media components,

temperature etc.) that is detrimental to the host cell, expression of the proteins may negatively effect the physiology of the host cell. In other cases overexpression of a desired gene may simply be unduly taxing for the producing cells. In such cases it is desirable to inhibit the expression of the desired gene until an optimal cell density has been achieved, and only then, 5 after an optimal period of cell culture *in vitro* or cell growth and development *in vivo* (determined empirically), induce gene expression in the cells to produce sufficient quantities of the protein. While a number of systems have been proposed and tried (as generally reviewed by Yarranton, G.T. 1992 Current Opinion in Biotechnology Vol. 3:506-511) many such systems do not allow for tight repression and subsequent complete activation. Others 10 employ impractical activation steps that are not expected to be useful in large scale fermentation or in whole animals. The current invention however fulfills all these criteria in eucaryotic expression systems using a transcriptional switch based on procaryotic control elements.

Aspects of the tightly regulatable genetic switch used in this invention for controlling 15 gene transcription are described in Gossen & Bujard, 1992, Proc. Natl. Acad. Sci. USA 89:55475551 and in US patent application Serial No. 08/076,726, entitled "Tight Control of Gene Expression in Eucaryotic Cells by Tetracycline-responsive Promoters" filed 14 June 1993, the full contents of both of which are incorporated herein by reference.

The genetic switch employed in this invention comprises two components: (i) a 20 polynucleotide (e.g. DNA) moiety encoding a tetracycline-controllable transcriptional activator (also referred to herein as a "transactivator" or tTA) and (ii) a gene of interest operably linked to, i.e., under the transcriptional control of, a promoter responsive to the transcriptional activator.

The tetracycline-controllable transactivator (tTA) is composed of a procaryotic tet 25 repressor (tetR) (also referred to as the first polypeptide) operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells (also referred to as the second polypeptide). Typically, nucleotide sequences encoding the first and second polypeptides are ligated to each other in-frame to create a chimeric gene encoding a fusion protein, although the first and second polypeptides can be operably linked by other means 30 that preserve the function of each polypeptide (e.g., chemically crosslinked). In one embodiment, the second polypeptide is a transcriptional activating protein such as the acidic transactivating domain of virion protein 16 (VP16) of herpes simplex virus (HSV) as in plasmids pUHD15-1 or pUHD151-1 (see Figure 11). It should be appreciated that other transactivators, including acidic, proline- or serine/threonine- or glutamine-rich 35 transactivating moieties as described below, may be substituted for the VP16 transactivator in the tetracycline-controllable fusion transactivator. In this embodiment, the second polypeptide of the fusion protein is capable of directly activating transcription.

In another embodiment, the second polypeptide of the tTA fusion protein indirectly activates transcription by recruiting a transcriptional activator to interact with the tetR fusion

protein. For example, tetR can be fused to a polypeptide domain (e.g., a dimerization domain) capable of mediating a protein-protein with a transcriptional activator protein, such as an endogenous activator present in a host cell. It has been demonstrated that functional associations between DNA binding domains and transactivation domains need not be 5 covalent (see e.g., Fields and Song (1989) *Nature* 340:245-247; Chien et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:9578-9582; Gyuris et al. (1993) *Cell* 75:791-803; and Zervos, A.S. (1993) *Cell* 72:223-232). Accordingly, the second polypeptide of the tTA fusion protein may not directly activate transcription but rather may form a stable interaction with an endogenous polypeptide bearing a compatible protein-protein interaction domain and transactivation 10 domain. Examples of suitable interaction (or dimerization) domains include leucine zippers (Landschulz et al. (1989) *Science* 243:1681-1688), helix-loop-helix domains (Murre, C. et al. (1989) *Cell* 58:537-544) and zinc finger domains (Frankel, A.D. et al. (1988) *Science* 240:70-73). Interaction of a dimerization domain present in the tTA fusion protein with an 15 endogeneous nuclear factor results in recruitment of the transactivation domain of the nuclear factor to the tTA, and thereby to a tet operator sequence to which the tTA is bound.

A variation of this approach is to construct a fusion of the tetR DNA binding sequence to the non-DNA binding amino acid sequences of the TATA binding protein (TBP) (TBP is described in Kao, C.C. et al. (1990) *Science* 248:1646-1650). The DNA binding form of TBP is part of a protein complex designated TFIID. The function of TBP in the 20 complex is to recruit other protein components of the TFIID complex to position near the transcription initiation site of eucaryotic genes containing a TATA box (i.e., TBP binding site). When bound to the TATA box, the TFIID complex subsequently mediates the sequential recruitment of other members of the basic transcriptional initiation complex, resulting in initiation of transcription (described in more detail in Buratowski, S. et al. (1989) *Cell* 56:549-561). Accordingly, when fused to tetR DNA binding sequences, TBP may 25 recruit other members of the basic transcription initiation complex to DNA sequences containing a tet operator(s). Furthermore, by substituting a TATA sequence present in a eukaryotic gene of interest with a tet operator(s), the tetR/TBP fusion protein can be targeted to this site in a manner dependent on the presence or absence of Tc (or analogue thereof), 30 resulting in Tc-dependent initiation of transcription. Since, in this approach, the gene of interest to be regulated by the tTA (i.e., tetR/TBP fusion protein) lacks a functional TATA element, the basal level of expression of the gene in the presence of Tc (or analogue) is expected to be very low. However, upon removal of Tc (or analogue), transcription initiation 35 is restored via binding of tetR/TBP to the tet operator(s) and recruitment of other components of the transcription initiation complex.

The tTA may be expressed in a desired host cell using otherwise conventional methods and materials by transfected or transforming the host cell with the tTA-encoding DNA operably linked to a conventional promoter (such as are mentioned elsewhere herein), e.g. for constitutive expression.

The second component of the genetic switch is the tTA-responsive transcriptional promoter to which the gene of interest is operably linked. The promoter may be a minimal promoter comprising, for example, a portion of the cytomegalovirus (CMV) IE promoter, operably linked to at least one tet operator sequence, derived for example from the 5 tetracycline resistance operon encoded in Tn10 of *E. coli* (Hillen & Wissmann, "Topics in Molecular and Structural biology" in Protein-Nucleic Acid Interaction, Saeger & Heinemann eds., Macmillan, London, 1989, Vol.10, pp.143-162), to serve as target sequences for a tTA.

Other suitable minimal promoters include PhCMV*-1, PhCMV*-2, and PtK*-1, 10 described herein, or other minimal promoters derived from promoter elements typically used in the cell line employed as described in the references throughout this application.

Minimal promoter elements particularly useful for a given cell line may be selected 15 from a series of deletion mutants of the original promoter nucleotide sequence, based on the ability of a given member of the series (for instance, placed as a Xhol/SacI fragment into the corresponding restriction sites of plasmid pUHC13-3) to be activated in transient transfection experiments using a cell line stably expressing the tetR-VP16 fusion protein; as will be appreciated a cell line stably expressing any other fusion of tetR with a protein domain capable of activating transcription (see below) can be used. As will also be appreciated 20 plasmid pUHC13-3 may be modified for the specific application by replacing genetic elements like poly-adenylation sites or splice sites with those functioning in the cell line in question. Specific details may be found in the references throughout this application or references cited therein, the full contents of which are incorporated herein by reference. A second criterion for the selection of the optimal minimal promoter is the degree of repression 25 in the presence of tetracycline (see below). Typically the deletion mutant with the highest activation factor as described below is chosen.

Promoter deletion mutants may be prepared as generally described by Rosen, C. et al 30 (1985) Cell Vol. 41, 813-823 or Nelson C. et al. (1986) Nature Vol. 322, 557-562. Other methods, including methods useful in the preparation of stable tetR-VP16 cell lines, are essentially as described in "Current Protocols in Molecular Biology" Ausubel, F.M. et al (eds.) 1989 Vol. 1 and 2 and all supplements to date Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, New York, the full contents of which are incorporated herein by reference, or as described in the other references cited throughout this application.

The presence of tet operator element(s) renders such recombinant promoter moieties 35 responsive to the tTA of the invention. In HeLa cells constitutively expressing the TetR-VP16 tTA, high levels of luciferase expression have been achieved under the control of such a modified CMV promoter sequence. The incorporation of the tetR domain within the tTA renders this expression system sensitive to the presence of tetracycline. The binding of tetracycline to the tetR domain of the tTA prevents the tTA from exerting its transactivating

effects. Depending on the concentration of tetracycline in the culture medium (0-1 µg/ml), the luciferase activity can be regulated up to five orders of magnitude in the previously mentioned example. This system provides both a reversible on/off switch and a differential control--as desired--for regulating gene expression in eucaryotic hosts. It should be
5 appreciated that tetracycline analogs which are capable of specific functional interaction with tetR may be used in place of tetracycline.

A eucaryotic production cell line of this invention is prepared according to the design described above. Assembly of the components and incorporation thereof into a eucaryotic host cell are conducted by otherwise conventional methods such as are described generally by
10 Kriegler, M. (editor), 1990, Gene Transfer and Expression, A Laboratory Manual (Stockton Press). Care should be taken to select for integration of the gene of interest into a chromosomal site that exhibits sufficiently low basal expression when, or to the extent, desired (see e.g. Table 1). The recombinant host cell obtained is grown in the presence of tetracycline or tetracycline analogues until an optimal density that has been determined
15 empirically to allow for subsequent induction of gene expression. After the desired cell density has been reached gene expression is induced by dilution and/or removal of the tetracycline or analog thereof. The culture is then continuously grown until a optimal expression level has been reached. The recombinant protein is then harvested according to standard procedures.

20 The use of eucaryotic cells as host cells for expression of recombinant proteins is generally reviewed in M.Kriegler 1990 "Gene Transfer and Expression, A Laboratory Manual". Stockton Press., incorporated herein as reference. While CHO^{dhfr}- cells (Urlaub, G. and Chasin (1980) Proc.Natl. Acad. Sci. USA 77: 4216-4220), 293 cells (Graham, F.L. et al. (1977) J. Gen.Viro. 36: pp. 59) or myeloma cells like SP2 or NSO (Galfre, G. and
25 Milstein, C. (1981) Meth Enzymol. 73 (B): 3-46) are commonly used it should be clear to those of ordinary skill in the art, that any eucaryotic cell line can be used in the practice of the subject invention, so long as the cell line is not incompatible with the protein to be expressed, the selection system chosen or the fermentation system employed. This invention is broadly applicable and encompasses non-mammalian eucaryotic cells as well, including insect (e.g.
30 Sp. frugiperda), yeast (e.g. S. cerevisiae, S. pombe, H. polymorpha) and fungal cells, containing and capable of expressing the two components of the foregoing genetic switch.

The eucaryotic host cells used for regulated expression in this invention may thus be yeast cells including, but not limited to Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces lactis and Hansenula polymorpha, as generally reviewed by Fleer, R. (1992).
35 Current Opinion in Biotechnology Vol. 3, No. 5: p. 486-496, the full contents thereof and of which the references cited therein are incorporated herein by reference.

In other embodiments the eucaryotic cells used for regulated expression are insect cells carrying in their chromosomes the heterologous DNA moiety encoding a transactivator fusion protein (tTA) comprising a tetracycline repressor and a protein capable of activating

transcription in the host cell. A second recombinant DNA moiety encoding the gene of interest operably linked to a promoter responsive to the transcriptional activator is carried on the baculovirus genome. Suitable general methods which may be used in the practice of these aspects of the invention are reviewed by O'Reilly et al. (1992) "Baculovirus expression vectors, A Laboratory Manual" Stockton Press, the full contents of which are incorporated herein by reference.

While the gene of interest may be a heterologous gene, i.e. not otherwise present in the parental host cell genome, an important aspect of this invention relates to the regulation of an endogenous gene of interest. In such cases the host cell is genetically engineered to insert 10 into the host cell genome the tTA-responsive promoter such that the desired endogenous gene is under the transcriptional control of the tTA-responsive promoter. This may be accomplished for example by linking a copy of the endogenous gene to the tTA-responsive promoter and transfecting or transforming the host cell with the recombinant construct. In one approach, the construct is introduced by homologous recombination into the loci of the 15 endogenous gene. Briefly, the tTA responsive promoter is flanked on the 5' side by sufficient DNA sequences from the upstream region but excluding the actual promoter region of the endogenous gene and on the 3'end by sequences representing the coding region of the endogenous gene. The extent of DNA sequence homology necessary for homologous recombination is discussed below.

In other approaches that construct is inserted at another genetic locus, either 20 predetermined or at random. In any case, the eucaryotic cell is also transformed or transfected with the DNA construct permitting expression of the tTA. Alternatively, the DNA construct encoding the tTA may itself be inserted at the locus of the endogenous gene of interest and the DNA moiety encoding the gene of interest operably linked to a 25 tTA-responsive promoter may be introduced elsewhere in the genome. In that embodiment, the tTA vector contains the tTA-encoding DNA moiety flanked by DNA sequence of the locus of the endogenous gene permitting homologous recombination of the construct into that locus.

The use of flanking DNA sequence to permit homologous recombination into a 30 desired genetic locus is known in the art. At present it is preferred that up to several kilobases or more of flanking DNA corresponding to the chromosomal insertion site be present in the vector on both sides of the tTA-encoding sequence (or any other sequence of this invention to be inserted into a chromosomal location by homologous recombination) to assure precise replacement of chromosomal sequences with the exogenous DNA. See e.g. 35 Deng et al, 1993, Mol. Cell. Biol 13(4):2134-40; Deng et al, 1992, Mol Cell Biol 12(8):3365-71; and Thomas et al, 1992, Mol Cell Biol 12(7):2919-23. It should also be noted that the eucaryotic cell of this invention may contain multiple copies of the gene of interest, e.g. by conventional genetic amplification, each operably linked to the tTA-responsive promoter.

It should be clear from the preceding that to achieve the goals of introducing the DNA moiety encoding the tTA into the host cell genome and of introducing the tTA-responsive promoter construct in operable linkage to the desired gene, vectors based on the following principles are required. First, to introduce the tTA-encoding construct into the genome of the host cell such that its expression will follow the regulated pattern of expression observed in the unmodified host cell for the gene of interest, it is necessary to introduce the tTA-encoding construct such that its expression is made subject to the transcription control elements associated with the gene of interest. One way to do so is to introduce the tTA-encoding construct by homologous recombination into the genetic locus of the gene of interest. A vector for such introduction comprises the DNA sequence encoding the tTA flanked by sufficient DNA sequence from the locus of the gene of interest in the host genome to permit the desired homologous recombination event in which the tTA and flanking DNA is effectively swapped for the flanking DNA copy and the DNA included therebetween within the host cell genome. As will be appreciated an expression construct containing a tTA responsive promoter operably linked to the DNA sequence of the endogenous gene can be integrated at random sites without the help of flanking homologous sequences as described in references throughout this application. Alternatively, to insert a DNA sequence comprising a tTA-responsive promoter or tetO control element(s) upstream of a desired gene, a construct is assembled in which the DNA comprising the tTA-responsive promoter is ligated upstream of a copy of the desired gene between DNA sequences flanking the desired insertion site in the host genome. In either event the tTA construct can be introduced as mentioned previously.

Using the foregoing genetic constructs and engineered eucaryotic cells, this invention further provides a method for regulating the expression of a gene of interest. In one aspect of this method eucaryotic host cells engineered as described above are cultured under otherwise conventional conditions suitable for cell growth and proliferation, but in a culture medium containing a substance capable of binding to the tetracycline repressor moiety and of blocking or inhibiting transcriptional activation. Tetracycline is the archetypical such substance. However, tetracycline analogs which bind to tetR to form a complex which is not transcriptionally activating may of course be substituted for tetracycline. The precise concentration of tetracycline or other such substance will depend on the substance's affinity for the tetR domain and/or the substance's specific inhibitory activity, as well as the cell density and copy number of the tTA and the desired level of inhibition of gene expression. Nonetheless, appropriate levels of inhibitory substance for the desired level of inhibition are readily determinable empirically without undue effort.

Cell culture in accordance with the preceding method negatively regulates, i.e. inhibits expression of the gene of interest, completely or partially. Culturing of the cells thereafter in media with a lower concentration (relative to the initial concentration) of the tetR binding substance permits gene expression to begin or to ensue at a now higher level. If an initial concentration of binding substance (e.g. tetracycline) is selected which is sufficient

to inhibit gene transcription substantially completely (e.g. transcription is not observed under conventional Northern blotting conditions), and in the following phase of cell culture the binding substance is substantially removed from the media, gene expression can be said to be regulated in an on/off manner. In some applications, intermediate levels of expression may be desired. To that end, concentrations of binding substance may be selected based on empirical data to provide predetermined intermediate level(s) of gene transcription. It should be understood that removal of the binding substance from the media may be effected by gradual, step-wise, continual or total replacement of culture media containing the binding substance with culture media lacking the binding substance or simply containing reduced levels of the binding substance.

Where the eucaryotic cells engineered in accordance with this invention are incorporated into the host organism, e.g. to create a transgenic organism, this invention provides a genetically engineered non-human animal capable of regulatably expressing a gene of interest. Such animal, in the broad sense, comprises cells containing and capable of expressing a heterologous DNA moiety encoding a tTA as previously defined and a DNA moiety comprising a gene of interest under the transcriptional control of a heterologous promoter responsive to the transcriptional activator.

Thus, this invention further relates to non-human animals derived by homologous recombination of one or more polynucleotide molecules of the invention into a specific target site within their genome, the offspring of such animals, as well as to a method to prevent or promote the expression of a targeted gene in a conditional manner.

This embodiment of the invention is able to solve a longstanding problem in the field generally described as gene targeting or gene knock out (Capechi. M.R. (1989) Science Vol 244, p. 1288-1292, Bradley, A. (1991) Current opinion in Biotechnology Vol. 2, p. 823-829) pertaining to genes whose mutations results in death of the homozygous embryos, e.g., as described for the knock out of the RB gene (Jacks, T. et al. (1992) Nature 359:295-300). If the genetic switch subject of the current invention is applied as described below, expression of an endogenous gene of interest operably linked to a tet operator sequence(s) can be stimulated by a tetracycline-controllable transactivator (tTA) of the invention and the animal develops like a nonmutated wildtype animal. Then, at a particular stage of development, expression of the endogeneous gene of interest can be switched off by raising the level of tetracycline or a tetracycline analogue in the circulation and the tissues of the animal by feeding or injecting the tetracycline or tetracycline analog to the animal, thereby inhibiting the activity of the tTA and transcription of the gene of interst. This method is generally referred to herein as a "conditional knockout".

As will be clear from the following, two principally different approaches have been devised to apply the genetic switch of this invention to the genome of the non-human animal in a way, that will allow for a temporally and spatially correct expression of the endogenous gene. In one approach, the two elements of the genetic switch are in separate locations in the

chromosome and require two integration steps, another one achieves the desired result in one step.

In the first step of one embodiment of the invention non-human animals are derived by homologous recombination of the DNA sequences of the tTA into a specific DNA site 5 containing the nucleotide sequences of an endogenous gene of interest in such a way that part or all of the coding sequence of the endogenous gene is replaced with the tTA gene. This can be accomplished (see Figure 11) in the following steps:

- (1) assembling a chimeric gene in which the sequence of the first (i.e. tTA) 10 polynucleotide molecule of the invention is flanked by DNA sequences from the gene of interest such that upon incorporation of the chimeric gene into the host genome, the DNA sequences that normally control the expression of the target gene are fused to and control expression of the DNA sequences for the tTA.
- (2) introducing this chimeric gene into an embryonic stem cell line from a species of interest and screening resultant candidate embryonic cell clones to identify and 15 recover those cells in which homologous recombination has taken place at the locus of interest.
- (3) introducing those recombinant cells so identified and recovered into a blastocyst 20 from the species of interest to yield a chimeric embryo.
- (4) implanting the chimeric embryo into the uteri of pseudopregnant recipient mothers to facilitate development and birth of a homologous recombinant offspring.

25 This process results in offspring whose genome contains the DNA sequence encoding the tTA inserted in place of the gene of interest such that the tTA DNA is expressed in a pattern similar or identical to that of the gene of interest. These processes and their results are collectively and commonly referred to as "gene knock-out". These techniques are well established and described in: Wood et al. Proc. Natl. Acad. Sci. 90:4582-4585, Simon et al. 30 Nature Genetics 1:92-97 & Soriano et al. Cell 64:693-702 and references therein, the full content of which are in their entirety incorporated herein by reference.

The second step in this embodiment of the invention relates to the preparation of a second transgenic animal which contains in its genome the gene of interest under 35 transcriptional control of the tetracycline (Tc) responsive promoter element. This can be accomplished using the following method:

- (1) A chimeric DNA sequence is prepared where a Tc responsive promoter element, comprising at least one tet operator and a minimal promoter) is cloned 5' of the DNA

sequences encoding the endogenous gene of interest. One way to accomplish this is to replace the luciferase coding sequence and all polyadenylation elements in the plasmids pUHC13-3 or pUHC13-4 with the DNA sequence containing the complete genomic coding sequence of the endogenous gene and sufficient 3' non coding sequence to allow for proper polyadenylation. As will be appreciated the DNA sequence encoding the endogenous gene can also be cDNA (cloned as an example in such a way that it replaces exactly the luciferase gene in pUHC13-3 or pUHC13-4) or any combination of genomic DNA and cDNA designed to provide the complete coding sequence, any regulatory elements that may reside in intron sequences or is not contained in its entirety in the cDNA and a polyadenylation signal or other elements typically associated with the endogenous gene. General cloning and DNA manipulation methods are described in references cited throughout this application.

(2) The chimeric DNA sequence (called also "the chimeric transgene") is injected into a fertilized egg which is implanted into a pseudopregnant recipient mother and allowed to develop into an adult animal. In particular, a few hundred DNA molecules are injected into the pro-nucleus of a fertilized one cell egg. The microinjected eggs are then transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster et al. (1986) Proc.Natl.Acad.Sci. USA Vol. 83:9065-9069, the full contents of which are incorporated by reference herein, that about 25 % of mice which develop will inherit one or more copies of the microinjected DNA. A protocol for constructing such transgenic animals (Brinster et al. Proc. Natl. Acad. Sci. 83:4432-4445, Crenshaw et al. Genes 3: Dev 9:959-972 and references cited therein) is a well established technique as is the breeding of recombinant and hybrid animals.

Breeding of animals resulting from the first and the second step of this embodiment of the invention produces offspring containing both the replaced gene of interest and the chimeric transgene. In a preferred embodiment, animals heterozygous for the knockout of the endogenous gene resulting from the first step of this embodiment of the invention (and instead expressing a tTA gene) are used for breeding with animals that are homozygous for the chimeric transgene resulting from the second step of this embodiment of the invention. The resulting offspring are analyzed by standard techniques, including tail-blot analysis described in references throughout this application, and animals homozygous for both traits are selected. Typically about 50 % of the offspring should carry both traits. In these animals, replacement of the coding sequences of the gene of interest with those of the DNA sequences of the tTA is such that the tTA is expressed in a temporal and spatial pattern similar or identical to that of the gene of interest and regulates in *trans* expression of the gene of interest now under transcriptional control of (i.e., operably linked to) the DNA sequences of the Tet

operator and minimal promoter inserted at its 5' end.

As will be appreciated, the particular breeding strategy depends on the nature of the gene of interest. If the "knock out" of the endogenous gene with the tTA coding sequence is not lethal and the overall plan is to create animals where the functions of the gene of interest in the adult can be studied in the "on" or "off" state, the animals from the first step of this embodiment of the invention can be bred to homozygosity and then bred with the homozygous mice from the second step.

In this combination, the gene of interest is regulated by the addition or subtraction of tetracycline or its analogs from the food or water supply of the animal as discussed below.

In another embodiment of the invention, embryonic stem (ES) cell technology is used to prevent or promote expression of a gene interest in a conditional manner (Figure 12). In the first step of this embodiment of the invention, a chimeric DNA sequence (commonly referred to as a chimeric transgene) consisting of the DNA sequences of the tet operator(s) and a suitable minimal promoter inserted 5' of the DNA sequences encoding a gene of interest is introduced by stable, non-homologous recombination into random sites in the ES cell genome. Co-introduced with this chimeric construct is a selectable marker that enables the selection of cell clones that have integrated DNA constructs from cells that have not. As will be appreciated, the feeder cells supporting the growth of the ES cells have to express the same resistance gene used for the selection step. As an example, if the selection marker chosen is the hygromycin resistance gene, the primary feeder layer cells used for the ES cell culture can be derived from an animal transgenic for the hygromycin resistance gene prepared according to standard procedures for the preparation of transgenic animals, as cited throughout this application. ES cell clones are selected for low basal expression of the chimeric transgene using customary detection methods, such as evaluating the mRNA levels of the transgene as described in "Current Protocols in Molecular Biology" Ausubel, F.M. et al (eds.) 1989 Vol. 1 and 2 and all supplements to date, Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, New York, the full contents of which are incorporated herein by reference, or as described in the other references cited throughout this application. Other methods to detect expression of the transgene may include activity assays or assays designed to detect protein expression. Low basal expression of the transgene is determined relative to untransfected cells. Alternatively, low basal expression of the tet operator-linked transgene can be evaluated in different tissues of animals derived from the embryonic stem cells. For example, ES cells can be transfected with the transgene in culture, and the clones expanded, selected and injected into blastocysts to create transgenic animals. After standard identification and breeding to create animals carrying the transgene in all tissues, the baseline expression of the tet-operator linked transgene can be examined in various tissues of interest (e.g., by conventional techniques for analyzing mRNA expression, such as Northern blotting, S1 nuclease mapping or reverse transcriptase-polymerase chain reaction). Additionally, basal expression of the transgene can be examined in primary

cultures of cells derived from various tissues of the animal (e.g., skin cells in culture).

A second criterion for the selection of the stable clone is the ability of the tet operator-linked transgene to respond to transient or stable expression of tTA upon transfection of a tTA expression plasmid like pUHD15-1 or pUHD151-1. As will be appreciated, these 5 plasmids are cited as examples only and others can be devised that expressed sufficient quantities of tTA in ES cells. The ability of tTA to induce expression of a tet-operator linked transgene stably transfected into an ES cell clone can be examined by supertransfected the ES cell clone with a tTA expression plasmid and assaying expression of the transgene. Alternatively, inducibility of a tet-operator linked transgene can be examined in cells derived 10 from various tissues of a transgenic animal carrying the transgene by preparing primary cultures of cells from the animal (e.g., skin cell cultures), transfecting the cells with a tTA expression plasmid and assaying expression of the transgene in the cells by standard techniques.

A clone fulfilling the criteria discussed above is selected and expanded in number. 15 This clone is then used as a recipient of a gene knock-out procedure consisting of the following steps:

- (1) flanking the sequences of a polynucleotide molecule encoding a tTA of the invention by DNA sequences from a second gene of interest such that the DNA 20 sequences that normally control the expression of the second target gene of interest are fused to and control expression of the DNA sequences of encoding the tTA;
- (2) introducing this chimeric gene into an embryonic cell line from a species of interest and modified as described above and screening candidate embryonic cell 25 clones for those in which homologous recombination has taken place at the locus of interest;
- (3) introducing those recombinant cells into blastocysts from the species of interest; and
- (4) implanting the chimeric embryo into the uteri of pseudopregnant recipient mothers to facilitate development and birth of a homologous recombinant animal.

This process results in offspring containing a replacement of the amino acid coding 35 sequences of the second gene of interest with those of the DNA sequences of the tTA such that the tTA encoding sequence is expressed in a temporal and spatial pattern similar to that of the endogenous second gene of interest. In this case, it is necessary to self cross the recombinant animals (or breed to homozygosity) so that both copies of the target sequence into which the tTA coding sequences have been integrated are interrupted. This procedure

also leads to homozygosity of the tet-operator linked transgene (i.e., animals homozygous for both components of the genetic switch described herein can be produced). These techniques are well established and described in: Wood et al. Proc. Natl. Acad. Sci. 90:4582-4585, Simon et al. Nature Genetics 1:92-97; and Soriano et al. Cell 64:693-702 and references therein.

5 In yet another embodiment of the invention, embryonic stem (ES) cell technology can again be used to prevent or promote expression of a gene of interest in a conditional manner using a single homologous recombination step that will result in the integrated copy shown in Figure 13. In this method, a DNA construct containing a fusion of the sequences that normally flank the endogenous gene of interest at the 5' end (and contain sequences commonly referred to as promoter sequences) are fused to the DNA sequences encoding the 10 tTA molecule. At the 3' end of the tTA coding sequence, DNA sequences encoding resistance to a selectable marker are typically included. For example, a neomycin resistance gene, which may be fused to either a constitutive regulatory element (e.g., a pPGK promoter as depicted in Figure 13A) or to a tet operator sequence(s) (as depicted in Figure 13B) can be 15 inserted at the 3' end of the tTA encoding sequence. When the selectable marker is operably linked to a tet operator sequence(s), its expression is regulated by the tTA (e.g., a resistance phenotype will be expressed in the absence but not the presence of Tc). Finally, 3' of the selectable marker sequences in this DNA construct are inserted the DNA sequences encoding the endogenous gene of interest, which are also fused to at least one tet operator sequence and 20 a minimal promoter.

Because in this configuration of the DNA molecule, conventionally called the targeting vector, the coding sequences of the tTA, the selectable marker and the endogenous gene of interest are all flanked by the sequences normally flanking the endogenous gene of interest, this DNA construct has the potential for homologous recombination with the locus 25 of the endogenous gene of interest upon its introduction into cells such as, but not limited to ES cells. Homologous recombination of this type alters the natural locus such that the gene of interest falls under the control of the tTA and consequently under regulation by the presence or absence of tetracycline or derivative thereof. The expression of the tTA protein, on the other hand, follows the normal pattern of expression of the gene of interest. 30 Recombinant ES cells of this type are then used to generate intact organisms as has been described (Wood et al. Proc. Natl. Acad. Sci. 90:4582-4585, Simon et al. Nature Genetics 1:92-97; and Soriano et al. Cell 64:693-702) which can in turn be bred to homozygosity.

As will be appreciated, the close proximity of the promoter elements in this particular configuration of the DNA construct used for homologous recombination may require special 35 consideration to insulate the downstream tet operator/minimal promoter operably linked to the endogenous gene from long range effects of the endogenous promoter operably linked to the tTA coding sequence to achieve the required low basal level expression of the endogenous gene. Some possible solutions are strong transcriptional terminators known to those of ordinary skill in the art, DNA elements that increase the distance between the

elements or others that limit the effect of enhancer sequences (e.g., transcriptional insulators, including matrix attachment regions), all of which are to be cloned alone or in combination in between the selectable marker expression unit (e.g., neomycin resistance gene with linked promoter) and the tTA-responsive transcriptional promoter sequence (see Figure 13).

5 Examples of suitable transcriptional terminators, transcriptional insulators, matrix attachment regions and/or other sequences which can be included in the "single hit" targeting vector to inhibit basal transcription of the tet operator-linked endogenous gene are described in Sato, K. et al. (1986) Mol. Cell. Biol. 6:1032-1043; Michel, D. et al. (1993) Cell. Mol. Biol. Res. 39:131-140; Chung, J.H. et al. (1993) Cell 74:505-514; Neznanov, N. et al. (1993) Mol. Cell. Biol. 13:2214-2223; and Thorey, I.S. et al. (1993) Mol. Cell. Biol. 13:6742-6751.

10

The different animals resulting from any of the above mentioned embodiments can be studied either in the absence (endogenous gene switched "on") or presence (endogenous gene switched "off") of tetracycline or tetracycline analogues as described for other transgenic animals below. Such animals can be used to identify, compare and characterize the activity of substances which interact with, upon or through the action of the gene product of interest.

15 The present invention relates to a control system that in eucaryotic cells allows regulation of expression of an individual gene over up to five orders of magnitude. This system is based on regulatory elements of a tetracycline resistance operon, e.g. Tn10 of *E. coli* (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in Protein-Nucleic Acid Interaction, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162), in which transcription of resistance-mediating genes is negatively regulated by the tetracycline repressor (tetR). In the presence of tetracycline or a tetracycline analogue, tetR does not bind to its operators located within the promoter region of the operon and allows transcription. By combining tetR with a protein capable of activating transcription in eucaryotes, e.g. the C-terminal domain of VP16 from HSV (known to be essential for the transcription of the immediate early vital genes (Triebenberg et al., (1988) Genes Dev. 2:718-729), a hybrid transactivator is generated that stimulates minimal promoters fused to tetracycline operator (tetO) sequences. These promoters are virtually silent in the presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to tetO sequences.

20 The specificity of the tetR for its operator sequence (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in Protein-Nucleic Acid Interaction, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162) as well as the high affinity of tetracycline for tetR (Takahashi et al., J. Mol. Biol. 187:341-348 (1986)) and the well-studied chemical and physiological properties of tetracyclines constitute a basis for an inducible expression system in eucaryotic cells far superior to the lacR/O/IPTG system. This has already been demonstrated in plant cells, in which direct repressor action at promoter sites is efficiently reversed by the antibiotic (Gatz & Quail, (1988) Proc. Natl. Acad. Sci. USA 85:1394-1397, Gatz et al., (1991) Mol. Gen. Genet. 227:229-237). However, these

previous systems used a tet repressor alone to inhibit gene expression, which may be inefficient or require high concentrations of the repressor intracellularly to function effectively. In contrast, the tTA of the present invention functions as a transcriptional activator to stimulate expression of a tet operator-linked gene.

5 In particular, the invention relates to a polynucleotide molecule coding for a transactivator fusion protein comprising the tet repressor (tetR) and a protein capable of directly or indirectly activating transcription in eucaryotes. The portion of the polynucleotide molecule coding for tetR may be obtained according to Altschmied et al., EMBO J. 7:4011-4017 (1988), the contents of which are fully incorporated by reference herein. Other
10 tetR sequences are available from Genbank and/or are disclosed in Waters, S.H. et al. (1983) Nucl. Acids Res. 11:6089-6105; Unger, B. et al. (1984) Gene 31:103-108, Unger, B. et al. (1984) Nucl. Acids Res. 12:7693-7703; Tovar, K. et al. (1988) Mol. Gen. Genet. 215:76-80; Hillen, W. and Schollmeier, K. (1983) Nucl. Acids Res. 11:525-539 and Postle, K. et al. (1984) Nucl. Acids Res. 12:4849-4863, the contents of each of which are fully incorporated
15 herein by reference.

The portion of the polynucleotide molecule coding for the negatively charged C-terminal domain of HSV-16, a protein known to be essential for transactivation in eucaryotes, may be obtained according to Triezenberg et al., Genes Dev. 2:718-729 (1988), the contents of which are fully incorporated by reference herein. Preferably, the activating
20 domain comprises the C-terminal 130 amino acids of the virion protein 16. Alternatively, other polypeptides with transcriptional activation ability in eucaryotic cells can be used in the tTA of the invention. Transcriptional activation domains found within various proteins have been grouped into categories based upon similar structural features. Types of transcriptional activation domains include acidic transcription activation domains, proline-rich transcription
25 activation domains, serine/threonine-rich transcription activation domains and glutamine-rich transcription activation domains. Examples of acidic transcriptional activation domains include the VP16 regions already described and amino acid residues 753-881 of GAL4. Examples of proline-rich activation domains include amino acid residues 399-499 of CTF/NF1 and amino acid residues 31-76 of AP2. Examples of serine/threonine-rich
30 transcription activation domains include amino acid residues 1-427 of ITF1 and amino acid residues 2-451 of ITF2. Examples of glutamine-rich activation domains include amino acid residues 175-269 of Oct1 and amino acid residues 132-243 of Sp1. The amino acid sequences of each of the above described regions, and of other useful transcriptional activation domains, are disclosed in Seipel, K. et al. (EMBO J. (1992) 13:4961-4968).

35 The polynucleotide molecule coding for tetR may be linked to a polynucleotide molecule coding for the activating domain (e.g., of HSV VP16) and recombined with vector DNA in accordance with conventional recombinant DNA techniques, including blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid

undesirable joining, and ligation with appropriate ligases. Alternatively, nucleic acid fragments encoding the repressor and the activating domain can be obtained by polymerase chain reaction amplification of appropriate nucleotide sequences using template DNA encoding either the repressor or the activating domain (e.g., encoding VP16). The amplified 5 DNA fragments can then be ligated such that the protein coding sequences remain in-frame and the chimeric gene so produced can be cloned into a suitable expression vector.

Preferably, the polynucleotide molecule coding for the transactivator fusion protein further comprises an operably linked promotor. The promotor may be an inducible promotor or a constitutive promotor. Examples of such promotors include the human cytomegalovirus 10 promotor IE as taught by Boshart et al., Cell 41:521-530 (1985), ubiquitously expressing promotors such as HSV-Tk (McKnight et al., Cell 37:253-262 (1984)) and β -actin promoters (e.g. the human β -actin promoter as described by Ng et al., Mol. Cell. Biol. 5:2720-2732 15 (1985)), as well as promoters in combination with control regions allowing integration site independent expression of the transgene (Grosveld et al., Cell 51:975-985 (1987)), as well as tissue specific promoters such as albumin (liver specific, Pinkert et al., Genes Dev. 1:268-277 20 (1987)), lymphoid specific promoters (Calame and Eaton, Adv. Immunol. 43:235-275 (1988)), in particular promoters of T-cell receptors (Winoto and Baltimore, EMBO J. 8:729-733 (1989)) and immunoglobulins; Banerji et al., Cell 33:729-740 (1983); Queen and Baltimore, ibid. 741-748), neuron specific promoters (e.g. the neurofilament promoter; Byrne 25 and Ruddle, Proc. Natl. Acad. Sci. USA 86:5473-5477 (1989)), pancreas specific promoters (Edlund et al., Science 230:912-916 (1985)) or mammary gland specific promoters (milk whey promoter, U.S. Patent No. 4,873,316 and European Application Publication No. 264,166) as well as developmentally regulated promoters such as the murine hox promoters (Kessel and Cruss, Science 249:374-379 (1990)) or the α -fetoprotein promoter (Campes and Tilghman, Genes Dev. 3:537-546 (1989)), the contents of each of which are fully 30 incorporated by reference herein. Preferably, the promoter is constitutive in the respective cell types. In one embodiment of the invention, the polynucleotide molecule encoding the transactivator is integrated at a predetermined location within a second target DNA molecule (e.g., a gene of interest within a chromosome) such that the tTA-coding sequences are placed under the control of endogenous regulatory elements (e.g., a 5' regulatory region of a target 35 gene of interest into which the tTA-coding sequence is integrated). Depending upon which gene the tTA-coding sequences are integrated into, the endogenous regulatory elements may provide constitutive expression of the tTA in many cell types or may limit expression of the tTA to a particular cell or tissue type.

The invention also relates to another polynucleotide molecule coding for a protein, wherein said polynucleotide is operably linked to a tTA-responsive promoter. Typically, this tTA-responsive promoter comprises a minimal promotor operatively linked to at least one tet operator (tetO) sequence. The tetO sequence may be obtained, for example, according to Hillen & Wissmann, "Topics in Molecular and Structural Biology," in Protein-Nucleic Acid

Interaction, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162, the contents of which are fully incorporated by reference herein. Other tetO sequences which may be used in the practice of the invention may be obtained from Genbank and/or are disclosed in Waters, S.H. et al. (1983) Nucl. Acids Res. 11:6089-6105; Hillen, W. and Schollmeier, K. (1983) Nucl. Acids Res. 11:525-539; Stüber, D. and Bujard, H. (1981) Proc. Natl. Acad. Sci. USA 78:167-171; Unger, B. et al. (1984) Nucl. Acids Res. 12:7693-7703; and Tovar, K. et al. (1988) Mol. Gen. Genet. 215:76-80, which are fully incorporated by reference herein in their entirety. One, two, three, four, five, six, seven, eight, nine or ten or more copies of the tet operator sequence may be employed, with a greater number of such sequences allowing an enhanced range of regulation. As shown in the Examples, multiple copies of the tet operator sequence provides a synergistic effect on the ability to control expression of the heterologous protein.

The polynucleotide sequence specifying the cytomegalovirus promotor may be obtained according to Boshart et al., Cell 41:521-530 (1985), the contents of which are fully incorporated by reference herein. Preferably, positions +75 to -53 to +75 to -31 of the promotor-enhancer are employed as a minimal promoter. The promotor may be followed by a polylinker and then by the gene coding for the protein of interest. While the luciferase gene or other reporter gene, e.g. the gene coding for chloramphenicol acetyltransferase or β -galactosidase, may be used to demonstrate the operability of the regulatory system, the invention is not intended to be so limited. Examples of such genes include, but are not limited to the estrogen receptor, the GABA receptor, the progesterone receptor and the X-protein of HBV.

The present invention also relates to eucaryotic cells transfected with the polynucleotide molecules of the present invention. In particular, the invention relates to eucaryotic cells transfected with

(a) a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic tet repressor and a protein capable of activation transcription in eucaryotes; and

(b) a second polynucleotide molecule coding for a protein, wherein said second polynucleotide molecule is operably linked to a minimal promotor and at least one tet operator sequence.

The two polynucleotide molecules may reside on the same or separate vectors. In a preferred embodiment, the first polynucleotide is integrated into the chromosome of a eucaryotic cell or transgenic animal and the second polynucleotide is introduced as part of a vector. Integration may be achieved where there is crossover at regions of homology shared between the incoming polynucleotide molecule and the particular genome.

The expression of the heterologous protein from such transfected eucaryotic cells may be tightly regulated. Unexpectedly, it has been determined that the expression system of the present invention may be used to regulate expression by about 5 orders of magnitude. In addition, it has been discovered that the expression system of the present invention allows one to rapidly turn "on" and "off" the expression of the heterologous gene in a reversible way. Moreover, it has been discovered that the expression system of the invention allows one to achieve a desired level of expression according to how much tetracycline or tetracycline analogue is employed (see Figure 3). Thus, the expression system of the present invention is a great advance in the art.

10 The invention also relates to transgenic animals comprising at least a first polynucleotide molecule of the present invention encoding a tTA. Such transgenic animals may be obtained, for example, by injecting the polynucleotide into a fertilized egg which is allowed to develop into an adult animal. In particular, a few hundred DNA molecules are injected into the pro-nucleus of a fertilized one cell egg. The microinjected eggs are then transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442 (1985), the contents of which are fully incorporated by reference herein, that about 25% of mice which develop will inherit one or more copies of the microinjected DNA. It is also possible to prepare a polynucleotide molecule comprising a milk protein promoter and microinject the 15 DNA into the fertilized egg to give, upon development, a transgenic mammal which is capable of producing the heterologous protein in its milk, when in the absence of tetracycline or a tetracycline analog. See International Application Publication No. WO 88/00239 and European Application Publication No. O264,166, the contents of which are fully incorporated by reference herein.

20

25 The invention also relates to non-human animals and their offspring derived by homologous recombination of the DNA sequences of the first polynucleotide molecules of the invention into a specific DNA site containing the nucleotide sequences of a gene referred to as the target gene. This would be accomplished in the following steps: 1) flanking the sequences of the first polynucleotide molecule of the invention encoding a tTA by DNA 30 sequences from the target site such that the DNA sequences that normally control the expression of the target gene are fused to and control the expression of the DNA sequences of the first polynucleotide molecules of the invention, 2) introducing this chimeric gene into an embryonic cell line from the species of interest and screening candidate embryonic cell clones for those in which homologous recombination has taken place at the target gene locus, 3) introducing those recombinant cells into a blastocysts from the species of interest, 4) implanting the chimeric embryo into the uteri of pseudopregnant recipient mothers to facilitate development and birth. This process will result in offspring containing a replacement of the amino acid coding sequences of the target gene with those of the DNA sequences of the first polynucleotide molecule of the invention such that this corresponding

amino acid sequence will be expressed in a pattern similar to that of the target gene. These processes and their results are collectively and commonly referred to as "gene knock-out". These techniques are well established and described in: Wood et al. *Proc. Natl. Acad. Sci.* 90:4582-4585, Simon et al. *Nature Genetics* 1:92-97 & Soriano et al. *Cell* 64:693-702 and references therein.

The invention also relates to a method to prevent or promote the expression of the target gene in a conditional manner. This may be accomplished by breeding an animal containing the target gene knock-out (as outlined in the preceding paragraph) with a transgenic animal derived by the following method. The transgenic animal would be 5 constructed by inserting, by micro-injection, a chimeric DNA sequence (commonly referred to as a chimeric transgene) consisting of the DNA sequences of the second polynucleotide molecule of the invention inserted 5' of the DNA sequences encoding the amino acid sequence of the target gene into the genome of a fertilized egg which is allowed to develop into an adult animal. The protocol for the construction of such transgenic animals is a well 10 established technique (Brinster et al. *Proc. Natl. Acad. Sci.* 83:4432-4445, Crenshaw et al. *Genes & Dev* 3:959-972 and references therein) as is the breeding of animals. From the breeding will result offspring containing both the gene knock-out and the chimeric transgene. That is, replacement of the amino acid coding sequences of the target gene with those of the 15 DNA sequences of the first polynucleotide molecule of the invention such that this corresponding amino acid sequence will be expressed in a pattern similar to that of the target gene and, the DNA sequences of the second polynucleotide molecule of the invention 20 inserted 5' of the DNA sequences encoding the amino acid sequence of the target gene. In this combination the target gene can be regulated by the addition or subtraction of tetracycline or its analogs from the food or water supply of the animal.

Thus, the invention also relates to a method to down regulate the expression of a protein coded for by a polynucleotide, comprising cultivating the transfected eucaryotic cells of the present invention in a medium comprising tetracycline or a tetracycline analogue. As 25 described in the Examples, it is possible to closely control the extent of expression by carefully controlling the concentration of tetracycline or tetracycline analogue in the culture media. As shown in Figure 3, panel A, as little as 0.0001 µg/ml of tetracycline will begin to result in a decrease of polypeptide (luciferase) expression. At about 0.1 µg/ml, the expression is essentially shut off. The concentration of tetracycline or tetracycline analogue which can be used to regulate the expression level may range from about 0.001 to about 1 µg/ml.

The invention also relates to a method to up regulate the expression of a protein coded 30 for by a polynucleotide, comprising cultivating the eucaryotic cells of the present invention in a medium lacking tetracycline or a tetracycline analogue.

The invention also relates to a method to use regulated gene expression in the production of recombinant proteins as generally reviewed by Yarranton, G.T 1992, the whole article incorporated as reference herein. Expression of recombinant proteins that are cytotoxic

or otherwise interfere with physiological processes in cells has been hampered by the lack of suitable methods to tightly regulate gene expression. In contrast, a production cell line according to the current invention is grown in the presence of tetracycline or tetracycline analogues until an optimal density (assessed empirically to allow for subsequent induction of gene expression) and expression is induced by dilution of the regulating compound. The culture is continuously grown until an optimal expression level has been reached. The recombinant protein is then harvested according to standard procedures.

As a preferred embodiment, eucaryotic cells are used for expression of recombinant proteins as generally reviewed in "Gene Transfer and Expression" (M. Kriegler 1990) incorporated herein as reference. While CHO^{dhfr}- cells (Urlaub, G. and Chasin, L. 1980),²⁹³ cells (Graham, F.L. et al. 1977) or myeloma cells like SP2 or NS0 (Galfre, C. and Milstein, C. 1981) are commonly used it should be clear to the skilled in the art, that any eucaryotic cell line can be used that is suitable for the protein to be expressed, the selection system chosen and the fermentation system employed.

In another preferred embodiment, the cells used for regulated expression are yeast cells including, but not limited to *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis* and *Hansenula polymorpha* as generally reviewed by Fleer, R. 1992, the whole article incorporated as referenced herein.

In another preferred embodiment, the cells used for regulated expression are insect cells with the gene and promoter region carried on the baculovirus genome as generally reviewed in "Baculovirus expression vectors" (O'Reilly et al. 1992), the whole document incorporated as referenced herein.

As can be appreciated, the tissue specificity of some promoters dictate that the tet operator sequence/promoter sequence fusion has to be designed with the particular application and cell line in mind following the teachings in this application using the promoters customarily used for the cell line in question; examples for those promoters are given in the relevant references mentioned above.

It should be clear from the foregoing that it is critical in the current invention that the production cell line is selected for a very low basal expression of the gene under control of the Tet operator/CMV promoter sequence. There are numerous methods currently available employing enzymatically assisted or unassisted homologous recombination to target repeatedly a chromosomal location found empirically to be suited for the integration of the gene encoding the recombinant protein. In addition to the homologous recombination approaches already described herein, enzyme-assisted site-specific integration systems are known in the art and can be applied to the components of the regulatory system of the invention to integrate a DNA molecule at a predetermined location in a second target DNA molecule. Examples of such enzyme-assisted integration systems include the Cre recombinase-lox target system (e.g., as described in Baubonis, W. and Sauer, B. (1993) *Nucl. Acids Res.* 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* 89:7905-

7909) and the FLP recombinase-FRT target system (e.g., as described in Dang, D.T. and Perrimon, N. (1992) Dev. Genet. 13:367-375; and Fiering, S. et al. (1993) Proc. Natl. Acad. Sci. USA 90:8469-8473).

Media which may be used in the practice of the invention include any media which 5 are compatible with the transfected eucaryotic cells of the present invention. Such media are commercially available (e.g. from Gibco/BRL).

Alternatively, it is possible to down regulate the expression of a protein in a transgenic animal of the present invention by administering to the animal tetracycline or tetracycline analogue. The tetracycline or tetracycline may be administered by any means that 10 achieves its intended purpose, e.g. by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route (see e.g., Example 2). The dosage administered will be dependent upon the age, health, and weight of the animal, kind of concurrent treatment, if any, and frequency of treatment. To up regulate the expression of the protein, the administration of 15 tetracycline or tetracycline analogue may then be interrupted.

The invention also relates to a kit comprising a carrier means having in close confinement therein at least two container means such as tubes, vials, bottles and the like, each of which containing a polynucleotide molecule which can be used in the practice of the invention. In particular, the invention relates to a kit comprising a carrier means having in 20 close confinement therein at least two container means, wherein a first container means contains a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic tet repressor and a protein capable of activation transcription in eucaryotes in a form suitable for homologous recombination; and a second container means contains a second polynucleotide molecule comprising a minimal promotor operably linked to at least 25 one tet operator sequence, wherein the second polynucleotide molecule is capable of being ligated to a heterologous gene sequence coding for a polypeptide and activating the expression of the heterologous protein.

The invention also relates to kits comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a 30 eucaryotic cell transfected with a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic tet repressor and a protein capable of activation transcription in eucaryotes in a form suitable for homologous recombination; and a second container means contains a second polynucleotide molecule comprising a minimal promotor operably linked to at least one tet operator sequence, wherein the second polynucleotide 35 molecule is capable of being ligated to a heterologous gene sequence coding for a polypeptide and activating expression of the polypeptide.

The invention is widely applicable to a variety of situations where it is desirable to be able to turn gene expression "on" and "off", or regulate the level of gene expression, in a rapid, efficient and controlled manner without causing pleiotropic effects or cytotoxicity.

The invention may be particularly useful for gene therapy purposes in humans, in treatments for either genetic or acquired diseases. The general approach of gene therapy involves the introduction of one or more nucleic acid molecules into cells such that one or more gene products encoded by the introduced genetic material are produced in the cells to restore or enhance a functional activity. For reviews on gene therapy approaches see Anderson, W.F. (1992) *Science* 256:808-813; Miller, A.D. (1992) *Nature* 357:455-460; Friedmann, T. (1989) *Science* 244:1275-1281; and Cournoyer, D., et al. (1990) *Curr. Opin. Biotech.* 1:196-208. However, current gene therapy vectors typically utilize constitutive regulatory elements which are responsive to endogenous transcriptions factors. These vector systems do not allow for the ability to modulate the level of gene expression in a subject. In contrast, the regulatory system of the invention provides this ability.

To use the system of the invention for gene therapy purposes, at least one DNA molecule is introduced into cells of a subject in need of gene therapy (e.g., a human subject suffering from a genetic or acquired disease) to modify the cells. The cells are modified to contain 1) nucleic acid encoding a tTA of the invention in a form suitable for expression of the tTA in the host cells and 2) a gene of interest (e.g., for therapeutic purposes) operatively linked to a tTA-responsive promoter (e.g., a *tet* operator sequence(s) and minimal promoter). Preferably, one or both of these DNA molecules is integrated into a predetermined location within a chromosome of the human cells by homologous recombination. A single DNA molecule encoding both components of the regulatory system of the invention can be used, or alternatively, separate DNA molecules encoding each component can be used. The cells of the subject can be modified *ex vivo* and then introduced into the subject or the cells can be directly modified *in vivo* by conventional techniques for introducing nucleic acid into cells. Expression of the gene of interest in the cells of the subject is stimulated in the absence of Tc or a Tc analogue, whereas expression is then inhibited by administering Tc or a Tc analogue to the patient. The level of gene expression can be varied depending upon which particular Tc analogue is used as the inducing agent. Additionally, expression of the gene of interest can be adjusted according to the medical needs of the individual, which may vary throughout the lifetime of the individual. Thus, the regulatory system of the invention offers the advantage over constitutive regulatory systems of allowing for modulation of the level of gene expression depending upon the requirements of the therapeutic situation.

Genes of particular interest to be expressed in cells of a subject for treatment of genetic or acquired diseases include those encoding adenosine deaminase, Factor VIII, Factor IX, dystrophin, β -globin, LDL receptor, CFTR, insulin, erythropoietin, anti-angiogenesis factors, growth hormone, glucocerebrosidase, β -glucuronidase, α 1-antitrypsin, phenylalanine hydroxylase, tyrosine hydroxylase, ornithine transcarbamylase, arginosuccinate synthetase, UDP-glucuronyl transferase, apoA1, MDR1 and MRP multidrug resistance genes, TNF, soluble TNF receptor, interleukins (e.g., IL-2), interferons (e.g., α - or γ -IFN) and other cytokines and growth factors.

Gene therapy applications of particular interest in cancer treatment include overexpression of a cytokine gene (e.g., TNF- α) in tumor infiltrating lymphocytes or ectopic expression of cytokines in tumor cells to induce an anti-tumor immune response at the tumor site), expression of an enzyme in tumor cells which can convert a non-toxic agent into a toxic agent, expression of tumor specific antigens to induce an anti-tumor immune response, expression of tumor suppressor genes (e.g., p53 or Rb) in tumor cells, expression of a multidrug resistance gene (e.g., MDR1 and/or MRP) in bone marrow cells to protect them from the toxicity of chemotherapy.

Gene therapy applications of particular interest in treatment of viral diseases include expression of trans-dominant negative viral transactivation proteins, such as trans-dominant negative tat and rev mutants for HIV or trans-dominant ICp4 mutants for HSV (see e.g., Balboni, P.G. et al. (1993) *J. Med. Virol.* 41:289-295; Liem, S.E. et al. (1993) *Hum. Gene Ther.* 4:625-634; Malim, M.H. et al. (1992) *J. Exp. Med.* 176:1197-1201; Daly, T.J. et al. (1993) *Biochemistry* 32:8945-8954; and Smith, C.A. et al. (1992) *Virology* 191:581-588), expression of trans-dominant negative envelope proteins, such as env mutants for HIV (see e.g., Steffy, K.R. et al. (1993) *J. Virol.* 67:1854-1859), intracellular expression of antibodies, or fragments thereof, directed to viral products ("internal immunization", see e.g., Marasco, W.A. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893) and expression of soluble viral receptors, such as soluble CD4.

The regulatory system of the invention can also be used to express a suicide gene (such as a ricin or HSV tk gene) in cells in a conditional manner to allow for destruction of the cells (e.g., *in vivo*) following a particular therapy. For example, a suicide gene can be introduced into tumor cells to be used for anti-cancer immunization or into the viral genome of a live attenuated viral to be used as a vaccine. The tumor cells or viral vaccine carrying the suicide gene are administered to a subject in the presence of Tc (or analogue thereof). Following immunization, the drug is withdrawn (e.g., administration is stopped), thereby inducing expression of the suicide gene to destroy the tumor cells or cells carrying the live virus.

Cells types which can be modified for gene therapy purposes include hematopoietic stem cells, myoblasts, hepatocytes, lymphocytes, airway epithelium and skin epithelium. For further descriptions of cell types, genes and methods for gene therapy see e.g., Wilson, J.M. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano, D. et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Wolff, J.A. et al. (1990) *Science* 247:1465-1468; Chowdhury, J.R. et al. (1991) *Science* 254:1802-1805; Ferry, N. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Wilson, J.M. et al. (1992) *J. Biol. Chem.* 267:963-967; Quantin, B. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Dai, Y. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; van Beusechem, V.W. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Rosenfeld, M.A. et al. (1992) *Cell* 68:143-155; Kay, M.A. et al. (1992) *Human Gene Therapy* 3:641-647; Cristiano, R.J. et al. (1993) *Proc. Natl. Acad. Sci. USA*

90:2122-2126; Hwu, P. et al. (1993) *J. Immunol.* 150:4104-4115; and Herz, J. and Gerard, R.D. (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816.

The regulatory system of the invention can also be used to produce and isolate a gene product (e.g., protein) of interest. Large scale production of a protein of interest can be accomplished using cultured cells *in vitro* which have been modified to contain 1) nucleic acid encoding a tTA of the invention in a form suitable for expression of the tTA in the host cells and 2) a gene of interest (e.g., encoding a protein of interest) operatively linked to a tTA-responsive promoter (e.g., a *tet* operator sequence(s) and minimal promoter). For example, mammalian, yeast or fungal cells can be modified to contain these nucleic acid components as described herein. Alternatively, an insect cell/baculovirus expression system can be used. To produce and isolate a gene product of interest, a host cell (e.g., mammalian, yeast or fungal cell) carrying the two components of the regulatory system of the invention (e.g., nucleic acid encoding a tTA and a gene of interest, encoding the gene product of interest, linked to a tTA-responsive promoter) are first grown in a culture medium in the presence of tetracycline or a tetracycline analogue. Under these conditions, expression of the gene of interest is repressed. Next, the concentration of tetracycline or the tetracycline analogue in the culture medium is reduced to stimulate transcription of the gene of interest. The cells are then further cultured in the absence of Tc (or analogue thereof) until a desired amount of the gene product encoded by the gene of interest is produced by the cells. The gene product can then be isolated from harvested cells or from the culture medium by standard techniques.

The invention also provides for large scale production of a protein of interest in animals, such as in transgenic farm animals. Advances in transgenic technology have made it possible to produce transgenic livestock, such as cattle, goats, pigs and sheep (reviewed in Wall, R.J. et al. (1992) *J. Cell. Biochem.* 49:113-120; and Clark, A.J. et al. (1987) *Trends in Biotechnology* 5:20-24). Accordingly, transgenic livestock carrying in their genome the components of the regulatory system of the invention can be constructed. Thus, by appropriate mating, double transgenic animals carrying a transgene encoding a tTA of the invention and a transgene comprising a tTA-responsive promoter linked to a gene of interest (the gene of interest may be either an exogenous or an endogenous gene) can be obtained. In the absence of Tc (or analogue), expression of the gene of interest is stimulated in the transgenic animals. By administering Tc (or analogue) to the animal, expression of the gene of interest can be inhibited. Protein production can be targeted to a particular tissue by linking the nucleic acid encoding the tTA to an appropriate tissue-specific regulatory element(s) which limits expression of the transactivator to certain cells. For example, a mammary gland-specific regulatory element, such as the milk whey promoter (U.S. Patent No. 4,873,316 and European Application Publication No. 264,166), can be linked to the tTA-encoding transgene to limit expression of the transactivator to mammary tissue. Thus, in the absence of Tc (or analogue), the protein of interest will be produced in the mammary tissue of

the transgenic animal, whereas protein expression can be downmodulated by administering Tc or a Tc analogue. The protein can be designed to be secreted into the milk of the transgenic animal, and if desired, the protein can then be isolated from the milk.

Having now generally described this invention, the same will be understood by reference to the following examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified. The contents of all publications, references, patents and published patent applications cited throughout the application are hereby incorporated by reference.

10 **Example 1: Regulation of Gene Expression in Cells by tTA
Materials and Methods**

Construction of the transactivators tTA and tTAS. The tetR sequence was originally recovered from pWH510 (Altschmied et al., EMBO J. 7:4011-4017 (1988), the disclosure of which is fully incorporated by reference herein) by PCR and inserted into pUHD10-1

15 (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5404 (1989)), resulting in pUHD14-1 (see, the Dissertation of Manfred Gossen, "Prokaryotic Repressor Operator Systems in the Control of Eucaryotic Gene Expression, Heidelberg University, 1993, the contents of which are fully incorporated by reference herein). A unique AflII cleavage site, overlapping the tetR stop codon in this plasmid construct, allows for the in-frame insertion of coding sequences.

20 To generate tTA, a 397-base-pair (bp) MluI/FokI fragment of pMSVP16 (Trienza et al., Genes Dev. 2:718-729 (1988), the disclosure of which is fully incorporated by reference herein), coding for the C-terminal 130 amino acids of VP16 of HSV, was blunted by filling in the protruding ends with T4 DNA polymerase. This DNA was inserted in pUHD14-1, previously cleaved with AflII, and blunted by mung bean nuclease. The resulting plasmid, 25 pUHD15-1, encodes the tTA sequence (Fig. 1, panel a) under the control of the PhCMV (human cytomegalovirus promoter IE; see below). In a homologous approach, a DNA fragment coding for the 97-amino acid C-terminal portion of VP16 was fused to tetR by PCR-mediated cloning. The resulting plasmid, pUHD151-1, encodes the smaller version of the trans-activator, tTAS (Fig. 1, panel a).

30

Construction of PhCMV* and the Luciferase Reporter Plasmid.

Plasmid pUHC13-1 is a derivative of pUHD10-1 (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5404 (1989)). It contains the promoter-enhancer sequence of PhCMV, spanning position +75 to position -675 (Boshart et al., Cell 41:521-530 (1985)). This 35 promoter is followed by a polylinker and the luciferase gene of Photinus pyralis fused to the SV40 small-t intron and poly(A) signal. The latter elements and the luciferase gene were transferred from pSV2L,ΔA5' (DeWit et al., Mol. Cell. Biol. 7:725-737 (1987)). By this transfer, the N-terminus of luciferase has been modified as described (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5404 (1989)). The enhancer region of PhCMV was removed

- 36 -

by PCR-mediated cloning, whereby a Xho I site was introduced adjacent to position -53. The resulting minimal promoter, PhCMV* (Fig. 1, panel b) is part of the reporter plasmid pUHC13-2.

5 Construction of PhCMV*-1 and PhCMV*-2.

To combine PhCMV* with tet operators, the 19-bp inverted repeat sequence of operator 02 of Tn10 (Triebenberg et al., Genes Dev. 2:718-729 (1988)) was synthesized as part of a 42-bp DNA fragment [SEQ ID NO: 10]:

(upper strand: 5' TCGAGTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAAG 3').

10 Upon annealing, the two complementary strands exposed the compatible protruding ends of a Xho I and a Sal I cleavage site at the 5 and 3' ends, respectively. Ligation of this fragment into the Xho I site of the polylinker of pT81-luc (Nordeen, S.K., BioTechniques 6:454-457 (1988)) created, upon cloning, single as well as multiple inserts of operator sequences upstream of a thymidine kinase (tk) minimal promoter from HSV contained in pT81-luc. The tk promoters containing one, two, and seven operator sequences were examined for their ability to be activated in transient expression experiments using the HeLa cell line HtTa-1 (see below). All constructs were active in tTA producing cells in a tetracycline-dependent manner. The heptameric version of the tetO sequences caused by far the highest activation of all Ptk-tetO constructs. It therefore was removed as a Xhol/Sal fragment and transferred into 15 pUHC13-2. Due to the asymmetric location of the tetO within the polylinker of pT81-luc, the resulting plasmids pUHC13-3 and pUHC13-4 contain the heptameric tetOs in two orientations differing in the distance between the operators and position + 1 of PhCMV by 19 bp. The two tetO-containing promoters were designated PhCMV*-1 and PhCMV*-2 (Fig. 1, panel b).

25

Band-Shift Assay.

Cytoplasmic and nuclear cell extracts from ~ 2 x 10⁶ cells were prepared as described by Andrews and Faller, Nucl. Acids Res. 19:2499 (1991), except that the cytoplasmic protein fraction was centrifuged once more (1 hr, 100,000 x g). Nuclear proteins were extracted by a 30 buffer containing 20 mM Hepes-KOH (pH 7.9), 25 % glycerol, 420 mM NaCL, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Aliquots (5 µl) of nuclear extracts were mixed with 15 µl of binding buffer (10 mM Tris HCl, pH 7.5/10 mM MgCl₂) containing 20 µg of calf thymus DNA, 5 µg of bovine serum albumin, and 2 fmol of ³²P-labeled tetO DNA. The tetO DNA was isolated from pUHC13-3 as a 42-bp Taq I fragment whose protruding ends were filled in by Klenow enzyme in the presence of [α -³²P]dCTP. After 20 min. at room temperature, aliquots of the binding reaction mixture were loaded onto a 5 % polyacrylamide/0.07 % bisacrylamide gel. Electrophoresis was carried out in 90 mM Tris base/90 mM boric acid/3 mM EDTA at 5 V/cm.

Luciferase Assays.

Cell grown to ~ 80 % confluence in 35-mm dishes in Eagle's minimum essential medium were washed with 2 ml of phosphate-buffered saline before they were lysed in 25
5 mM Tris phosphate, pH 7.8/2 mM dithiothreitol/2 mM diaminocyclohexanetetraacetic acid/10 % glycerol/1 % Triton X-100 for 10 min at room temperature. The lysate was scraped off the culture dishes and centrifuged for 10 sec in an Eppendorf centrifuge. Next, aliquots (10 µl) of the supernatant were mixed with 250 µl of 25 mM glycylglycine/15 mM MgSO₄/5 mM ATP and assayed for luciferase activity in a Lumat LB9501 (Berthold, Wildbad,
10 F.R.G.) using the integral mode (10 sec). D-Luciferin (L6882, Sigma) was used at 0.5 mM. The background signal measured in extracts of HeLa cells that did not contain a luciferase gene was indistinguishable from the instrumental background [80-120 relative light units (rlu)/10 sec]. Protein content of the lysates was determined according to Bradford (Bradford, M.M., Anal. Biochem. 72:248-254 (1976)).

15

RESULTSConstruction and Characterization of the tTA.

To convert the prokaryotic tet repressor into a eucaryotic transactivator, it was fused to the negatively charged C-terminal domain of HSV-VP16, known to be essential for transactivation. (Triebenberg et al., Genes Dev. 2:718-729 (1988)). Sequences coding for either a 97- or a 127-amino acid C-terminal portion of VP16 were fused to the tetR gene, resulting in the coding sequences of tTAS and tTA, respectively (Fig. 1, panel a). In plasmids coding for tTA (pUHD15-tTAs (pUHD 151 -1), the transactivator sequences are flanked upstream by PhCMV and downstream by the SV40 poly(A) site. The two fusion proteins did not differ in their functional *in vivo* properties.

HeLa cells transiently transfected with pUHD15-1 produced a fusion protein of the expected molecular mass (37 kDa), as demonstrated in immunoblots of the electrophoretically separated cytoplasmic and nuclear extracts (Fig. 2, panel a). When nuclear extracts were mixed with the tetO DNA, the electrophoretic mobility of the DNA was diminished. The specificity of the interaction between tTA and operator DNA was confirmed by the finding that no mobility change for tetO DNA was detectable in the presence of the specific inducer tetracycline (Fig 2, panel b).

Construction of a tTA-Dependent Promoter.

35 To generate promoters activatable by tTA, tetOs were inserted upstream of minimal promoter sequences. For PhCMV, the upstream enhancer region was removed by PCR and a Xho I cleavage site was introduced adjacent to position -53. This minimal promoter, designated PhCMV*, spans the original PhCMV sequence from +75 to -53 (+ 1 being the first nucleotide transcribed) and, in addition, contains a Stu I site around -31 (Fig. 1, panel b).

tetO sequences were fused to this core promoter by insertions at the Xho I site (Fig. 1).

The tetO sequence 02 of Tn10 is a 19-bp inverted repeat to which tetR binds as a 46-kDa dimer (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in Protein-Nucleic Acid Interaction, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162). It was chemically synthesized and ligated into the Xho I cleavage site of the polylinker located upstream of the minimal tk promoter in plasmid pT81-luc (Nordeen, S.K., BioTechniques 6:454-457 (1988)). Multiple insertions of tetOs created a set of promoters that contained between 1 and 7 tetO sequences upstream from position -81 of the tk promoter. A Xho I/Sal I fragment containing 7 tetOs, fused head to tail, was recovered from one of the constructs and transferred into the Xho I site upstream of PhCMV*. Due to the asymmetry of the Xho I/Sal I fragment, two PhCMV*-tetO constructs were obtained that differ in the distance between the operators and position +1 of PhCMV, which is 95 bp for PhCMV*-1 and 76 bp for PhCMV*-2. The plasmids containing these promoters are designated pUHC13-3 and pUHC13-4, respectively (Fig. 1, panel b). When HeLa cells were transiently transfected with these plasmids, high levels of luciferase activity were monitored whenever the cells were cotransfected with pUHD15-1, which provided the coding sequence of tTA. Little activity was observed with cultures grown in the presence of tetracycline (1.0 µg/ml) or with plasmids containing PhCMV* only. Since PhCMV*-1 and PhCMV*-2 were activated by tTA to a significantly higher degree than any of the Ptk constructs, the latter ones were not investigated further.

Quantitation of PhCMV*-1 and PhCMV*-2 Activation by tTA.

To quantify the stimulation of PhCMV*-tetO constructs by tTA, HeLa cell lines were established that contained the PhCMV*-1- or the PhCMV*-2-luciferase, as well as the PhCMV-tTA expression units stably integrated. Conditions for culturing and selecting cells have been described (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5405 (1989)). In a first step, cells were cotransfected with pUHD15-1 and pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1:327-341 (1982)). Clones resistant to G418 were assayed for transactivation of PhCMV*-1 by transient transfection with pUHC13-3. In all HeLa cell clones in which the tetracycline-responsive promoters were active, tTA was not detectable by Western blots or by immunofluorescence. Its presence was just barely visible in electrophoretic mobility shift experiments of highly labeled tetO DNA. This indicates very low intracellular concentrations of tTA and may reflect a selection against squelching effects caused by higher concentrations of VP16-activating domains (Gill & Ptashne, Nature (London) 334:721-724 (1988)).

One of the positive clones, HtTA-1, was then cotransfected with a plasmid carrying the hygromycin-resistance gene (pHMR272; Bernard et al., Exp. Cell Res. 158:237-243 (1985)) and either pUHC13-3 or pUHC13-4, resulting in the X and T series of clones, respectively. Clones resistant to hygromycin and G418 were assayed for luciferase activity. As shown in Table 1 below, in the absence of tetracycline, this activity differed in individual

clones by almost four orders of magnitude. However, in all cases, the luciferase activity was sensitive to tetracycline in the culture. This demonstrates that the expression of luciferase is dependent on the function of tTA, which obviously is capable of activating promoter constructs PhCMV*-1 and PhCMV*-2.

5

Table 1.
Tetracycline-dependent Luciferase Activity of
Different HeLa Cell Clones

| Clone | With Tc | Without Tc | Activation Factor |
|-------|-----------|------------------|-------------------------|
| T7 | 1074 ± 75 | 79,197 ± 2,119 | 7.3 × 10 ¹ |
| T11 | 2.5 ± 0.4 | 34,695 ± 1,127 | 1.3 × 10 ⁴ |
| T12 | 3.5 ± 0.9 | 35,298 ± 5,009 | 1 × 10 ⁴ |
| T14 | ≤2 | 33 ± 4 | ≥ 1.5 × 10 ¹ |
| T15 | 286 ± 47 | 49,070 ± 2,784 | 1.7 × 10 ² |
| T16 | ≤2 | 541 ± 133 | ≥ 2.7 × 10 ² |
| X1 | ≤2 | 257,081 ± 40,137 | ≥ 2.7 × 10 ⁵ |
| X2 | ≤2 | 104,840 ± 20,833 | ≥ 5 × 10 ⁴ |
| X7 | 75 ± 7 | 125,745 ± 18,204 | 1.6 × 10 ³ |

The HeLa cell clone HtTA-1, which constitutively expresses tTA, was cotransfected with pUHC13-3 or pUHC13-4 and pHMR272.

Hygromycin-resistant clones were examined for luciferase activity. Nine clones identified were subcloned and luciferase activity was quantified in the presence (1 μ/ml) and absence of tetracycline (Tc). Values are arithmetic means of three independent luciferase determinations (from three independently grown cultures). Luciferase activities of <2 rlu/μg of protein are too close to the instrumental background to be quantified.

When the luciferase activity within various clones was monitored in the presence and absence of tetracycline hydrochloride (Sigma), two remarkable results emerged. (i) In all clones tested, tTA greatly stimulated promoter activity, even up to five orders of magnitude in clone X1. (ii) In clones T14, T16, X1 and X2 (Table 1), tetracycline reduced luciferase activity to values that cannot be quantified even at high protein concentration of extracts due to instrumental limitations (i.e., rlu/μg of protein >2). This demonstrates that PhCMV*-1 and PhCMV*-2 are virtually silent when integrated in the proper genomic environment and that their activity depends exclusively on the action of tTA.

10

The tTA inactivation studies were carried out with 1 μg of tetracycline per ml in the culture medium. A partial inactivation of tTA is, however, readily achieved with tetracycline concentrations below 0.1 μg/ml, as shown in Fig. 3, panel a. In the two clones analyzed (T12 and X1), a stepwise reduction of the tetracycline concentration in the medium gradually increased the luciferase activity. These results again demonstrate that, in the case of clone

X1, tTA can regulate transcriptional activity, as monitored by luciferase activity, by over five orders of magnitude. Moreover, at tetracycline concentrations sufficient for full inactivation of tTA (0.1 µg/ml), no change in growth behavior or morphology of HeLa cells occurs. Only at tetracycline concentrations well above 10 µg/ml were such changes observed upon
5 prolonged incubation.

Kinetics of Tetracycline Action.

The time course of tetracycline action was analyzed in cultures grown in the absence or presence of tetracycline. At time 0, the antibiotic was added to the tetracycline-free
10 cultures (final concentration, 1 µg/ml), whereas the tetracycline-containing cultures were rinsed and incubated in fresh antibiotic-free medium (Fig. 3 panel b). At various times, cells were harvested and analyzed for luciferase activity. As shown in Fig. 3 panel b, the depletion of tetracycline leads to a rapid induction of luciferase activity reaching > 20 % of the fully induced level within 12 hr. A similarly rapid reduction of luciferase activity was observed
15 when tetracycline was added to the fully active tetracycline-free system: within 8 hr activity dropped to about 10 % and reached < 2 % of its original value after 12 hr.

The fusion of the Tn10-derived *E. coli* tetR with the activation domain of VP16 from HSV has generated a transactivator exhibiting all of the properties required for the specific and stringent regulation of an individual gene in a eucaryotic cell. The transactivator tTA
20 produced in HeLa cells binds specifically to tetO sequences *in vitro*. This association is prevented by tetracycline. When bound to tetOs placed upstream of minimal promoters, tTA efficiently activates transcription from such promoters *in vivo* in a tetracycline-dependent manner. The transactivator is produced in HeLa cells in amounts sufficiently high for strong activation of transcription though low enough to avoid any detectable squelching effects (Gill
25 & Ptashne Nature (London) 334:721-724 (1988)).

The usefulness of heterologous regulatory systems as the one described here depends decisively on quantitative parameters such as the extent of inactivation and the efficiency of activation of gene expression as well as the kinetics of transition from one state to the other. For the tet system, these parameters were measured in HeLa cell lines that constitutively
30 express tTA and that also contain the luciferase gene stably integrated and under the control of tTA-dependent promoters. The clones characterized thus far express the luciferase gene to various extents. This is not surprising since differences in the integration sites and in the number of integrated transcription units would be expected. However, in all cases, the expression of luciferase is sensitive to tetracycline. In some clones, tetracycline has the most dramatic effect of reducing the luciferase activity from high levels over several orders of magnitude to background. This demonstrates that in HeLa cells, the two promoters PhCMV*-1 and PhCMV*-2, have no measurable intrinsic activity. Their function strictly depends on tTA. The residual luciferase activity observed in some clones in the presence of tetracycline must therefore be due to position effects.

The tTA-dependent promoters can be kept in a partially activated state by low concentrations of tetracycline. As shown in Fig. 3 panel a, varying the tetracycline concentration between 0 and 0.1 µg/ml allows adjustment of promoter activity within a range of several orders of magnitude. This may allow assessment also of quantitative parameters of gene function *in vivo*.

5 The activation and inactivation of tTA by the antibiotic appears to be not only an efficient but also a rapid process. When cells from tetracycline containing medium are shifted to tetracycline-free medium, significant luciferase activity is induced within 4 hr and > 20 % of the steady-state level is reached within 12 hr after the shift. Interestingly, even the 10 cultures that were only exposed to tetracycline-free medium during the washing procedure before reincubation in tetracycline-containing medium show a small but reproducible increase in luciferase activity that is still detectable after 4 hr (Fig. 3b).

When tetracycline is added to a culture of X1 cells, luciferase activity is reduced ~ 10-fold within 8 hr and > 50 fold within 12 hr. This decrease is remarkably fast if one 15 takes into account the half-life of luciferase of around 3 hr reported for eucaryotic cells (measured by cycloheximide inhibition: Ilguyen et al., J. Biol. Chem. 264:10487-10492 (1989); Thompson et al., Gene 103:171-177 (1991)) and indicates a rapid uptake of tetracycline by HeLa cells followed by a fast and efficient shutdown of transcription. Although the half-life of luciferase and its mRNA remains to be determined in this system, 20 these conclusions are supported by observations in plant cells, where tetracycline inactivates tetR within < 30 min (Gatz et al., Mol. Gen. Genet. 227:229-237 (1991)).

Taken together, these data show that tetracycline, unlike IPTG in a eucaryotic lacR/O-based system, is able to act fast in cultures of eucaryotic cells. The possibility of rapidly switching the activity of a tTA-dependent promoter not only is of interest in studying 25 gene function itself but also should allow analysis of mRNA decay rates of individual genes under physiological conditions.

In clone X1, tetracycline reduces luciferase activity reproducibly by five orders of magnitude. This suggests that binding of tetracycline to tTA may lower the association constant between the transactivator and its operator to a much greater extent than that 30 measured for tetR (Takahasi et al., J. Mol. Biol. 187:341-348 (1986)) and as described for IPTG in the lacR/O system, where the binding constant k_{RO} is reduced only 1000-fold by the inducer (Barkley and Bourgeois in The Operon, Miller and Reznikoff (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1980; pp. 177-220.)

On the other hand, the results obtained in transient experiments with minimal tk 35 promoters fused to single, dimeric, and heptameric tetO sequences strongly suggest a synergistic effect of multiple tTA binding sites. The efficient inactivation of tTA by tetracycline is therefore most likely due to a large difference in the binding constants of tTA and tTA/tetracycline for the tetO and the nonlinear effect of tetracycline interfering with a cooperative process.

In conclusion, the results indicate that promoter-activating systems as described here are most promising for regulating individual genes in higher eucaryotic cells for several reasons. (i) For activators, in particular when acting through a cooperative mechanism, intracellular concentrations can be kept low, ensuring an efficient inactivation by the effector--in this case, tetracycline. By contrast, repressors in general compete directly with transcription factors and/or RNA polymerases for binding within a promoter region. In the absence of cooperativity, however, the window at which the repressor concentration is sufficiently high for tight expression but still low enough for efficient induction may be narrow and not easily adjustable in different systems. (ii) In an activating system, as described here, the synthesis of tTA can be driven by a tissue-specific promoter, whereas the tTA-dependent promoters are expected to function tissue independently, since they may require only general transcription factors in addition to tTA. By contrast, in a repressor-based system in which operators have to be placed within the context of a promoter sequence, an influence on promoter specificity cannot be excluded. (iii) The tet system offers specific advantages when compared to the intensely studied lac system. For example, tetR binds tetracycline much tighter ($k_a \sim 10^9 M^{-1}$; Takahashi et al., J. Mol. Biol. 187:341-348 (1986)) than lacR complexes IPTG ($k_a 10^6 M^{-1}$; Barkley & Bourgeois in The Operon, Miller & Reznikoff, eds., Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1980, pp. 177-220). Thus, very low, nontoxic concentrations of tetracycline function effectively. Moreover, a large number of tetracycline analogues are known, of which some appear to have far superior properties as effectors than tetracycline itself. In this context, it is interesting to note that detailed information on the pharmacological properties of tetracycline, in particular pharmacokinetic parameters, is available, which will facilitate application of this system in transgenic animals.

25

Example 2: Regulation of Gene Expression in Transgenic Animals by tTA

To examine the ability of tTA to regulate gene expression *in vivo*, transgenic strains of mice were constructed which contained heterologous chromosomal insertions of either a tTA expression unit or a tTA-responsive promoter operably linked to a reporter gene. Single transgenic strains containing either the tTA expression unit or the tTA-responsive reporter unit were then cross bred and double transgenic progeny were identified. The double transgenic animals were then characterized as to the ability of tTA, in a tetracycline dependent manner, to regulate expression of the reporter gene. This example demonstrates that tTA effectively stimulates the expression of a gene operably linked to a tTA responsive promoter in multiple tissues of the animals *in vivo* in the absence of tetracycline (or analogue), whereas expression of the tTA-responsive gene is effectively inhibited in multiple tissues of the animals when tetracycline or an analogue thereof is administered to the animals. These results demonstrate that the tetracycline-controlled transcriptional regulatory system described herein functions effectively in animals, in addition to cell lines *in vitro*.

Generation of mice transgenic for a PhCMV-tTA expression unit

Mice expressing tTA protein were obtained by pronuclear injection into fertilized oocytes of a 2.7kb Xhol-PfmI fragment excised from plasmid pUHG15-1. This DNA fragment contained the tTA gene (shown in SEQ ID NO: 1) under the transcriptional control of the human CMV IE promoter (position +75 to -675) together with a rabbit β -globin polyadenylation site including an intron. The human CMV IE promoter is a constitutive promoter that allows expression of the tetR-VP16 fusion protein in many cell lines where chromosomal integration of the DNA sequence encoding tTA has occurred and is known to be functional in a variety of tissues in transgenic mice. DNA was injected into fertilized oocytes at a concentration of approximately 5 ng per μ l by standard techniques. Transgenic mice were generated from the injected fertilized oocytes according to standard procedures. Transgenic founder mice were analyzed using polymerase chain reaction (PCR) and Southern hybridization to detect the presence of the tTA transgene in chromosomal DNA of the mice.

Generation of mice transgenic for the PhCMV*-1 luciferase reporter unit

Mice carrying a PhCMV*-1 1uc reporter gene expression unit were generated by pronuclear injection into fertilized oocytes of a 3.1 kb XhoI-EaeI fragment excised from plasmid pUHC13-3. This DNA-fragment contains the luciferase gene under transcriptional control of the tetracycline-responsive PhCMV*-1 promoter (SEQ ID NO: 5), together with a SV40 t early polyadenylation site including an intron. DNA was injected into oocytes at a concentration of approximately 5 ng per μ l and transgenic mice were generated according to standard procedures. Transgenic founder mice were analyzed using Southern hybridization to detect the presence of the PhCMV*-1 1uc transgene in chromosomal DNA of the mice.

Generation of mice transgenic for the PhCMV*-1 luc and PhCMV tTA

Having constructed single transgenic mice expressing tTA or carrying PhCMV*-1 luc, double transgenic mice carrying both the tTA expression vector and the luciferase reporter-units were obtained through cross breeding of heterozygous mice transgenic for one of the two transgenes. Double transgenic animals were identified by standard screenings (e.g., PCR and/or Southern hybridization) to detect the presence of both the tTA transgene and the PhCMV*-1 1uc transgene in chromosomal DNA of the mice.

Induction and analysis of luciferase activity in tissue samples from mice

For oral administration, tetracycline or its derivative doxycycline were given in the drinking water at a concentration of 200 μ g per ml with 5 % sucrose to hide the bitter taste of the antibiotics. For lactating mice, the concentration was 2 mg per ml with 10 % sucrose to ensure a sufficient uptake via the milk by the young.

To analyze luciferase activity, mice were killed by cervical dislocation and tissue

samples were homogenized in 2 ml tubes containing 500 µl lysis-buffer (25 mM Tris phosphate, pH 7.8/2 mM DTT/ 2 mM EDTA/ 10 % glycerol/ 1 % Triton X100) using a Ultra-Turrax. The homogenate was frozen in liquid nitrogen and centrifuged after thawing for 5 min at 15,000g. 2-20 µl of the supernatant were mixed with 250 µl luciferase assay buffer (25 mM glycylglycine, pH 7.5/ 15 mM MgSO₄/ 5 mM ATP) and luciferase activity was measured for 10 sec after the injection of 100µl of a 125 µM luciferin solution using Berthold Lumat LB 9501. The protein concentration of the homogenate was determined using Bradford assay and luciferase activity was calculated as relative light units (rlu) per µg of total protein.

10

Results

Mice from 4 lines carrying the PhCMV-tTA transgene (CT1 through CT4) were mated with mice from line L7, transgenic for PhCMV*-1 luc. This line shows a very low but significant background of luciferase activity in different organs that is probably due to position effects at the integration site. The luciferase activity in different tissues of the double transgenic mice, either in the presence or absence of the tetracycline analogue doxycycline, is illustrated graphically in Figure 14. High luciferase activity was detectable in five tissues of the double transgenic mice examined: heart, muscle, pancreas, thymus and tongue. The tissue pattern of activated luciferase levels (i.e., in the absence of doxycycline) in the double transgenic mice was similar to expression patterns of the hCMV IE promoter reported in the literature. This is consistent with expression of the luc reporter gene being regulated by tTA (which is expressed in the mice under the control of the hCMV IE promoter). After administration of doxycycline to the mice for 7 days, luciferase activity was reduced close to background levels observed in single transgenic mice carrying only the PhCMV*-1 luc reporter unit (i.e., the L7 line). Depending on the individual animals used for comparison of induced and non-induced luciferase level, regulation factors up to 10,000 fold can be estimated e.g. in the pancreas. These results indicate that the tetracycline-controlled transcriptional regulatory system described herein can be used to efficiently regulate expression of genes in transgenic animals.

30

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

35

- 45 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: BASF Aktiengesellschaft
5 (C) CITY: 6700 Ludwigshafen
(D) STATE: Rheinland-Pfalz
(E) COUNTRY: Federal Republic of Germany

10 (i) APPLICANT/INVENTORS: Bujard, Hermann
Gossen, Manfred
Salfeld, Jochen G.
Voss, Jeffrey W.

15 (ii) TITLE OF INVENTION: Tight Control of Gene Expression in
Eucaryotic Cells by Tetracycline-Responsive
Promoters

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

20 (A) ADDRESSEE: Lahive & Cockfield
(B) STREET: 60 State Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: USA
25 (F) ZIP: 02109-1875

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
30 (D) SOFTWARE: ASCII text

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
35 (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US08/076,327
(B) FILING DATE: 14-JUN-1993

(viii) ATTORNEY/AGENT INFORMATION:

40 (A) NAME: DeConti, Giulio A., Jr.
(B) REGISTRATION NUMBER: 31,503
(C) REFERENCE/DOCKET NUMBER: BBI-013CPPC

(ix) TELECOMMUNICATION INFORMATION:

45 (A) TELEPHONE: (617) 227-7400
(B) TELEFAX: (617) 227-5941

INFORMATION FOR SEQ ID NO:1:

- 47 -

| | | | | |
|----|---|-----|-----|-----|
| | AAT AAG GCT AAA AGT TTT AGA TGT GCT TTA CTA AGT CAT CGC GAT GGA Asn Lys Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly 85 | 90 | 95 | 288 |
| 5 | GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA GAA AAA CAG TAT GAA ACT Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr 100 | 105 | 110 | 336 |
| 10 | CTC GAA AAT CAA TTA GCC TTT TTA TGC CAA CAA GGT TTT TCA CTA GAG Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu 115 | 120 | 125 | 384 |
| 15 | AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT ACT TTA GGT TGC Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys 130 | 135 | 140 | 432 |
| 20 | GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr 145 | 150 | 155 | 480 |
| 25 | CCT ACT ACT GAT AGT ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu 165 | 170 | 175 | 528 |
| 30 | TTT GAT CAC CAA GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu 180 | 185 | 190 | 576 |
| 35 | ATC ATA TGC GGA TTA GAA AAA CAA CTT AAA TGT GAA AGT GGG TCC GCG Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser Ala 195 | 200 | 205 | 624 |
| 40 | TAC AGC CGC GCG CGT ACG AAA AAC AAT TAC GGG TCT ACC ATC GAG GGC Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser Thr Ile Glu Gly 210 | 215 | 220 | 672 |
| 45 | CTG CTC GAT CTC CCG GAC GAC GCC CCC GAA GAG GCG GGG CTG GCG Leu Leu Asp Leu Pro Asp Asp Asp Ala Pro Glu Glu Ala Gly Leu Ala 225 | 230 | 235 | 720 |
| 50 | GCT CCG CGC CTG TCC TTT CTC CCC GCG GGA CAC ACG CGC AGA CTG TCG Ala Pro Arg Leu Ser Phe Leu Pro Ala Gly His Thr Arg Arg Leu Ser 245 | 250 | 255 | 768 |
| 55 | ACG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG GAC GAT TTC GAT Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp 260 | 265 | 270 | 816 |
| 60 | GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp 275 | 280 | 285 | 864 |
| 65 | CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro 290 | 295 | 300 | 912 |
| 70 | CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe 305 | 310 | 315 | 960 |

- 48 -

GAG CAG ATG TTT ACC GAT CCC CTT GGA ATT GAC GAG TAC GGT GGG TAG 1008
 Glu Gln Met Phe Thr Asp Pro Leu Gly Ile Asp Glu Tyr Gly Gly
 325 330 335

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 335 amino acids
- (B) TYPE: amino acid
- 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu
 1 5 10 15

15 Leu Asn Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln
 20 25 30

20 Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys
 35 40 45

25 Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His
 50 55 60

25 Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg
 65 70 75 80

30 Asn Lys Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly
 85 90 95

35 Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr
 100 105 110

Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu
 35 115 120 125

40 Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys
 130 135 140

45 Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr
 145 150 155 160

Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu
 165 170 175

45 Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu
 180 185 190

50 Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser Ala
 195 200 205

Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser Thr Ile Glu Gly
 210 215 220

55 Leu Leu Asp Leu Pro Asp Asp Asp Ala Pro Glu Glu Ala Gly Leu Ala

- 49 -

225 230 235 240

Ala Pro Arg Leu Ser Phe Leu Pro Ala Gly His Thr Arg Arg Leu Ser
245 250 255

5

Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp
260 265 27010 Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp
275 280 285Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro
290 295 30015 His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
305 310 315 320Glu Gln Met Phe Thr Asp Pro Leu Gly Ile Asp Glu Tyr Gly Gly
325 330 335

20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 894 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

30

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Herpes Simplex Virus
- (B) STRAIN: K12, KOS
- (C) INDIVIDUAL ISOLATE: tTAS transactivator

35

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 1..894

40

(ix) FEATURE:

- (A) NAME/KEY: mRNA
- (B) LOCATION: 1..894

45

(ix) FEATURE:

- (A) NAME/KEY: misc. binding
- (B) LOCATION: 1..207

50

(ix) FEATURE:

- (A) NAME/KEY: misc. binding
- (B) LOCATION: 208..297

55

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..891

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

- 50 -

| | | |
|----|---|-----|
| | ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu 1 5 10 15 | 48 |
| 5 | CTT AAT GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG Leu Asn Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln 20 25 30 | 96 |
| | AAG CTA GGT GTA GAG CAG CCT ACA TTG TAT TGG CAT GTA AAA AAT AAG Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys 35 40 45 | 144 |
| 10 | CGG GCT TTG CTC GAC GCC TTA GGC ATT GAG ATG TTA GAT AGG CAC CAT Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His 50 55 60 | 192 |
| 15 | ACT CAC TTT TGC CCT TTA GAA GGG GAA AGC TGG CAA GAT TTT TTA CGT Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg 65 70 75 80 | 240 |
| | AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA CTA AGT CAT CGC GAT GGA Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly 85 90 95 | 288 |
| 20 | GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA GAA AAA CAG TAT GAA ACT Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr 100 105 110 | 336 |
| | CTC GAA AAT CAA TTA GCC TTT TTA TGC CAA CAA GGT TTT TCA CTA GAG Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu 115 120 125 | 384 |
| 25 | AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT ACT TTA GGT TGC Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys 130 135 140 | 432 |
| 30 | GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr 145 150 155 160 | 480 |
| | CCT ACT ACT GAT AGT ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu 165 170 175 | 528 |
| 35 | TTT GAT CAC CAA GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu 180 185 190 | 576 |
| | ATC ATA TGC GGA TTA GAA AAA CAA CTT AAA TGT GAA AGT GGG TCT GAT Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser Asp 195 200 205 | 624 |
| 40 | CCA TCG ATA CAC ACG CGC AGA CTG TCG ACG GCC CCC CCG ACC GAT GTC Pro Ser Ile His Thr Arg Arg Leu Ser Thr Ala Pro Pro Thr Asp Val 210 215 220 | 672 |
| 45 | AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC GAG GAC GTG GCG ATG GCG Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala 225 230 235 240 | 720 |

- 51 -

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| CAT | GCC | GAC | GCG | CTA | GAC | GAT | TTC | GAT | CTG | GAC | ATG | TTG | GGG | GAC | GGG | 768 | |
| His | Ala | Asp | Ala | Leu | Asp | Asp | Phe | Asp | Leu | Asp | Met | Leu | Gly | Asp | Gly | | |
| | | | | 245 | | | | | 250 | | | | 255 | | | | |
| GAT | TCC | CCG | GGT | CCG | GGG | TTT | ACC | CCC | CAC | GAC | TCC | GCC | CCC | TAC | GGC | 816 | |
| 5 | Asp | Ser | Pro | Gly | Pro | Gly | Phe | Thr | Pro | His | Asp | Ser | Ala | Pro | Tyr | Gly | |
| | | | | 260 | | | | 265 | | | | 270 | | | | | |
| GCT | CTG | GAT | ATG | GCC | GAC | TTC | GAG | TTT | GAG | CAG | ATG | TTT | ACC | GAT | GCC | 864 | |
| Ala | Leu | Asp | Met | Ala | Asp | Phe | Glu | Phe | Glu | Gln | Met | Phe | Thr | Asp | Ala | | |
| | | | | 275 | | | 280 | | 285 | | | | | | | | |
| 10 | CTT | GGA | ATT | GAC | GAG | TAC | GGT | GGG | TTC | TAG | | | | | | 894 | |
| | Leu | Gly | Ile | Asp | Glu | Tyr | Gly | Gly | Phe | | | | | | | | |
| | | | | 290 | | | 295 | | | | | | | | | | |

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 297 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

| | | | | | | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| 20 | Met | Ser | Arg | Leu | Asp | Lys | Ser | Lys | Val | Ile | Asn | Ser | Ala | Leu | Glu | Leu | |
| | 1 | | | | | 5 | | | | 10 | | | 15 | | | | |
| 25 | Leu | Asn | Glu | Val | Gly | Ile | Glu | Gly | Leu | Thr | Thr | Arg | Lys | Leu | Ala | Gln | |
| | | | | 20 | | | | 25 | | | | 30 | | | | | |
| 30 | Lys | Leu | Gly | Val | Glu | Gln | Pro | Thr | Leu | Tyr | Trp | His | Val | Lys | Asn | Lys | |
| | | | | 35 | | | | 40 | | | 45 | | | | | | |
| 35 | Arg | Ala | Leu | Leu | Asp | Ala | Leu | Ala | Ile | Glu | Met | Leu | Asp | Arg | His | His | |
| | | | | 50 | | | 55 | | | 60 | | | | | | | |
| 40 | Thr | His | Phe | Cys | Pro | Leu | Glu | Gly | Glu | Ser | Trp | Gln | Asp | Phe | Leu | Arg | |
| | | | | 65 | | | 70 | | | 75 | | | 80 | | | | |
| 45 | Asn | Asn | Ala | Lys | Ser | Phe | Arg | Cys | Ala | Leu | Leu | Ser | His | Arg | Asp | Gly | |
| | | | | | 85 | | | | 90 | | | 95 | | | | | |
| 50 | Ala | Lys | Val | His | Leu | Gly | Thr | Arg | Pro | Thr | Glu | Lys | Gln | Tyr | Glu | Thr | |
| | | | | 100 | | | | 105 | | | 110 | | | | | | |
| 55 | Leu | Glu | Asn | Gln | Leu | Ala | Phe | Leu | Cys | Gln | Gln | Gly | Phe | Ser | Leu | Glu | |
| | | | | 115 | | | | 120 | | | 125 | | | | | | |
| 60 | Asn | Ala | Leu | Tyr | Ala | Leu | Ser | Ala | Val | Gly | His | Phe | Thr | Leu | Gly | Cys | |
| | | | | 130 | | | 135 | | | 140 | | | | | | | |
| 65 | Val | Leu | Glu | Asp | Gln | Glu | His | Gln | Val | Ala | Lys | Glu | Glu | Arg | Glu | Thr | |
| | | | | 145 | | | 150 | | | 155 | | | 160 | | | | |
| 70 | Pro | Thr | Thr | Asp | Ser | Met | Pro | Pro | Leu | Leu | Arg | Gln | Ala | Ile | Glu | Leu | |

- 52 -

| | | | |
|----|---|-----|-----|
| | 165 | 170 | 175 |
| | Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu | | |
| | 180 | 185 | 190 |
| 5 | Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser Asp | | |
| | 195 | 200 | 205 |
| 10 | Pro Ser Ile His Thr Arg Arg Leu Ser Thr Ala Pro Pro Thr Asp Val | | |
| | 210 | 215 | 220 |
| | Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala | | |
| | 225 | 230 | 235 |
| 15 | Asp Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly | | |
| | 245 | 250 | 255 |
| 20 | Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly | | |
| | 260 | 265 | 270 |
| | Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp Ala | | |
| | 275 | 280 | 285 |
| 25 | Leu Gly Ile Asp Glu Tyr Gly Gly Phe | | |
| | 290 | 295 | |

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 450 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

- 35 (ii) MOLECULE TYPE: DNA (genomic)

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Human cytomegalovirus
 - (B) STRAIN: K12, Towne

- 40 (ix) FEATURE:
 - (A) NAME/KEY: mRNA
 - (B) LOCATION: 382..450

- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

| | |
|--|-----|
| GAATTCCCTCG AGTTTACCA C TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC | 60 |
| ACTCCCTATC AGTGATAGAG AAAAGTGAAA GTCGAGTTA CCACCTCCCTA TCAGTGATAG | 120 |
| AGAAAAGTGA AAGTCGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG | 180 |
| TTTACCACTC CCTATCAGTG ATAGAGAAAA GTGAAAGTCG AGTTTACCA C TCCCTATCAG | 240 |
| 50 TGATAGAGAA AAGTGAAAGT CGAGTTTACC ACTCCCTATC AGTGATAGAG AAAAGTGAAA | 300 |
| GTCGAGCTCG GTACCCGGGT CGAGTAGGCG TGTACGGTGG GAGGCCTATA TAAGCAGAGC | 360 |

- 53 -

TCGTTTAGTG AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTG ACCTCCATAG 420
AAGACACCGG GACCGATCCA GCCTCCGCGG 450

(2) INFORMATION FOR SEQ ID NO:6:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 450 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human cytomegalovirus
(B) STRAIN: Towne

15 (ix) FEATURE:
(A) NAME/KEY: mRNA
(B) LOCATION: 382..450

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCCCTCG ACCCGGGTAC CGAGCTCGAC TTTCACTTTT CTCTATCACT GATAGGGAGT 60
GGTAAACTCG ACTTTCACTT TTCTCTATCA CTGATAGGGA GTGGTAAACT CGACTTTCAC 120
TTTCTCTAT CACTGATAGG GAGTGGTAAA CTCGACTTTC ACTTTCTCT ATCACTGATA 180
GGGAGTGGTA AACTCGACTT TCACTTTCT CTATCACTGA TAGGGAGTGG TAAACTCGAC 240
25 TTTCACTTTT CTCTATCACT GATAGGGAGT GGTAAACTCG ACTTTCACTT TTCTCTATCA 300
CTGATAGGGA GTGGTAAACT CGAGTAGGCG TGTACGGTGG GAGGCCTATA TAAGCAGAGC 360
TCGTTTAGTG AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTG ACCTCCATAG 420
AAGACACCGG GACCGATCCA GCCTCCGCGG 450

(2) INFORMATION FOR SEQ ID NO:7:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 398 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Herpes Simplex Virus
(B) STRAIN: KOS

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGCTCGACT TTCACTTTTC TCTATCACTG ATAGGGAGTG GTAAACTCGA CTTCACTTT 60

- 54 -

| | | | | | | | |
|------------|------------|------------|------------|------------|------------|------------|-----|
| TCTCTATCAC | TGATAGGGAG | TGGTAAACTC | GACTTTCACT | TTTCTCTATC | ACTGATAGGG | 120 | |
| AGTGGTAAAC | TCGACTTTCA | CTTTCTCTA | TCACTGATAG | GGAGTGGTAA | ACTCGACTTT | 180 | |
| CACTTTCTC | TATCACTGAT | AGGGAGTGGT | AAACTCGACT | TTCACTTTTC | TCTATCACTG | 240 | |
| ATAGGGAGTG | GTAAACTCGA | CTTCACTTT | TCTCTATCAC | TGATAGGGAG | TGGTAAACTC | 300 | |
| 5 | GAGATCCGGC | GAATTCGAAC | ACGCAGATGC | AGTCGGGCG | GCGCGGTCCG | AGGTCCACTT | 360 |
| | CGCATATTAA | GGTGACGCGT | GTGGCCTCGA | ACACCGAG | | | 398 |

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6244 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

15 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Human cytomegalovirus
- (B) STRAIN: Towne (hCMV)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pUHD BGR3

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

| | | | | | | |
|---------------|------------|------------|-------------|------------|------------|-----|
| CTCGAGTTTA | CCACTCCCTA | TCAGTGATAG | AGAAAAGTGA | AAGTCGAGTT | TACCACTCCC | 60 |
| TATCAGTGAT | AGAGAAAAGT | GAAAGTCGAG | TTTACCACTC | CCTATCAGTG | ATAGAGAAAA | 120 |
| GTGAAAGTCG | AGTTTACAC | TCCCTATCG | TGATAGAGAA | AAGTGAAAGT | CGAGTTTACC | 180 |
| ACTCCCTATC | AGTGATAGAG | AAAAGTGAAG | GTCGAGTTA | CCACTCCCTA | TCAGTGATAG | 240 |
| 25 AGAAAAGTGA | AAGTCGAGTT | TACCACTCCC | TATCAGTGAT | AGAGAAAAGT | GAAAGTCGAG | 300 |
| CTCGGTACCC | GGTCGAGTA | GGCGTGTACG | GTGGGAGGCC | TATATAAGCA | GAGCTCGTT | 360 |
| AGTGAACCGT | CAGATCGCCT | GGAGACGCCA | TCCACGCTGT | TTTGACCTCC | ATAGAAGACA | 420 |
| CCGGGACCGA | TCCAGCCTCC | GGGGCCCCGA | ATTCGAGCTC | GGTACCGGGC | CCCCCTCGA | 480 |
| GGTCGACGGT | ATCGATAAGC | TTGATATCGA | ATTCAGGAG | GTGGAGATCC | GGGGGTCCAG | 540 |
| 30 CCAAACCCCA | CACCCATTTC | CTCCTCCCTC | TGCCCTATA | TCCCGGCACC | CCCTCCTCCT | 600 |
| AGCCCTTTC | CTCCTCCCGA | GAGACGGGGG | AGGAGAAAAG | GGGAGTTCA | GTCGACATGA | 660 |
| CTGAGCTGAA | GGCAAAGGAA | CCTCGGGCTC | CCCACGTGGC | GGCGGGCGCG | CCCTCCCCCA | 720 |
| CCGAGGTGG | ATCCCAGCTC | CTGGGTCGCC | CGGACCCCTGG | CCCCCTCCAG | GGGAGCCAGA | 780 |
| CCTCAGAGGC | CTCGTCTGTA | GTCTCCGCCA | TCCCCATCTC | CCTGGACGGG | TTGCTCTTCC | 840 |

- 55 -

| | | |
|----|--|------|
| | CCCGGCCCTG TCAGGGGCAG AACCCCCAG ACGGGAAGAC GCAGGACCCA CCGTCGTTGT | 900 |
| | CAGACGTGGA GGGCGCATTT CCTGGAGTCG AAGCCCCGGA GGGGGCAGGA GACAGCAGCT | 960 |
| | CGAGACCTCC AGAAAAGGAC AGCGGCCCTGC TGGACAGTGT CCTCGACACG CTCTGGCGC | 1020 |
| | CCTCGGGTCC CGGGCAGAGC CACGCCAGCC CTGCCACCTG CGAGGCCATC AGCCCGTGGT | 1080 |
| 5 | GCCTGTTGG CCCCGACCTT CCCGAAGACC CCCGGGCTGC CCCCGCTACC AAAGGGGTGT | 1140 |
| | TGGCCCCGCT CATGAGCCGA CCCGAGGACA AGGCAGGCGA CAGCTCTGGG ACGGCAGCGG | 1200 |
| | CCCACAAAGGT GCTGCCAGG GGACTGTCAC CATCCAGGCA GCTGCTGCTC CCCTCCTCTG | 1260 |
| | GGAGCCCTCA CTGGCCGGCA GTGAAGCCAT CCCCGCAGCC CGCTGCGGTG CAGGTAGACG | 1320 |
| | AGGAGGACAG CTCCGAATCC GAGGGCACCG TGGGCCGCT CCTGAAGGGC CAACCTCGGG | 1380 |
| 10 | CACTGGGAGG CACGGCGGCC GGAGGAGGAG CTGCCCCCGT CGCGTCTGGA GCGGCCGCAG | 1440 |
| | GAGGCCTCGC CCTTGTCCCC AAGGAAGATT CTCGCTTCTC GGCGCCCGAG GTCTCCTTGG | 1500 |
| | CGGAGCAGGA CGCGCCGGTG GCGCCTGGC GCTCCCCGCT GGCCACCTCG GTGGTGGATT | 1560 |
| | TCATCCACGT GCCCATCCTG CCTCTCAACC ACGCTTCTC GGCCACCCGC ACCAGGCAGC | 1620 |
| | TGCTGGAGGG GGAGACCTAC GACGGGGGGG CGCGGGCGC CAGCCCCCTTC GTCCCGCAGC | 1680 |
| 15 | GGGGCTCCCC CTCTGCCTCG TCCACCCCTG TGGCGGGCGG CGACTTCCCC GACTGCACCT | 1740 |
| | ACCCGCCCCA CGCCGAGCCC AAAGATGACG CGTTCCCCCT CTACGGCGAC TTCCAGCCGC | 1800 |
| | CCGCCCTCAA GATAAAGGAG GAGGAAGAAG CGGCCGAGGC CGCGGCCGCGC TCCCCCGCTA | 1860 |
| | CGTACCTGGT GGCTGGTGCA AACCCCGCCG CCTTCCCGGA CTTCCAGCTG GCAGCGCCGC | 1920 |
| | CGCCACCCCTC GCTGCCGCT CGAGTCCCT CGTCCAGACC CGGGGAAGCG GCGGTGGCGG | 1980 |
| 20 | CCTCCCCAGG CAGTGCCTCC GTCTCCTCCT CGTCCTCGTC GGGTCGACC CTGGAGTGCA | 2040 |
| | TCCTGTACAA GGCAGAAGGC GCGCCGCCCC AGCAGGGCCC CTTCGCCCG CTGCCCTGCA | 2100 |
| | AGCCTCCGGG CGCCGGCGCC TGCGCTGC CGCGGGACGG CCTGCCCTCC ACCTCCGCC | 2160 |
| | CGGGCGCAGC CGCCGGGGCC GCCCCTGCAGC TCTACCCGAC GCTCGGCCTC AACGGACTCC | 2220 |
| | CGCAACTCGG CTACCAGGCC GCCGTGCTCA AGGAGGGCCT GCCGCAGGTC TACACGCC | 2280 |
| 25 | ATCTCAACTA CCTGAGGCCG GATTCAAGAAG CCAGTCAGAG CCCACAGTAC AGCTTCGAGT | 2340 |
| | CACTACCTCA GAAGATTTGT TTGATCTGTG GGGATGAAGC ATCAGGCTGT CATTATGGTG | 2400 |
| | TCCTCACCTG TGGGAGCTGT AAGGTCTTCT TTAAAAGGGC AATGGAAGGG CAGCATAACT | 2460 |
| | ATTTATGTGC TGGAAAGAAAT GACTGCATTG TTGATAAAAT CGCAGGAAA AACTGCCGG | 2520 |
| | CGTGTGCCT TAGAAAGTGC TGTCAAGCTG GCATGGCTT TGGAGGGCGA AAGTTAAAAA | 2580 |
| 30 | AGTTCAATAA AGTCAGAGTC ATGAGAGCAC TCGATGCTGT TGCTCTCCCA CAGCCAGTGG | 2640 |

- 56 -

| | | |
|----|---|------|
| | GCATTCCAAA TGAAAGCAA CGAACACTT TTTCTCCAAG TCAAGAGATA CAGTTAATT | 2700 |
| | CCCCCTCTAAT CAACCTGTTA ATGAGCATTG AACCGAGATGT GATCTATGCA GGACATGACA | 2760 |
| | ACACAAAGCC TGATACCTCC AGTTCTTGC TGACGAGTCT TAATCAACTA GGCGAGCGGC | 2820 |
| | AACTTCTTTC AGTGGTAAAA TGTTCCAAAT CTCTTCCAGG TTTTCGAAAC TTACATATTG | 2880 |
| 5 | ATGACCAGAT AACTCTCATC CAGTATTCTT GGATGAGTTT AATGGTATTG GGACTAGGAT | 2940 |
| | GGAGATCCTA CAAACATGTC AGTGGCAGA TGCTGTATTT TGCACCTGAT CTAATATTAA | 3000 |
| | ATGAACAGCG GATGAAAGAA TCATCATTCT ATTCACTATG CCTTACCATG TGGCAGATAC | 3060 |
| | CGCAGGAGTT TGTCAAGCTT CAAGTTAGCC AAGAAGAGTT CCTCTGCATG AAAGTATTAC | 3120 |
| | TACTTCTTAA TACAATTCTT TTGGAAGGAC TAAGAAGTCA AAGCCAGTTT GAAGAGATGA | 3180 |
| 10 | GATCAAGCTA CATTAGAGAG CTCATCAAGG CAATTGGTTT GAGGCAAAAA GGAGTTGTTT | 3240 |
| | CCAGCTCACA GCGTTTCTAT CAGCTCACAA AACTTCTTGA TAACTTGCAT GATCTTGTC | 3300 |
| | AACAACTTCA CCTGTACTGC CTGAATACAT TTATCCAGTC CCGGGCGCTG AGTGTGAAAT | 3360 |
| | TTCCAGAAAT GATGTCTGAA GTTATTGCTG CACAGTTACC CAAGATATTG GCAGGGATGG | 3420 |
| | TGAAACCAC TCTCTTCAT AAAAAGTGAA TGTCAATTAT TTTTCAAAGA ATTAAGTGT | 3480 |
| 15 | GTGGTATGTC TTTCGTTTTG GTCAGGATTA TGACGTCTCG AGTTTTATA ATATTCTGAA | 3540 |
| | AGGGAATTCC TGCAGCCCGG GGGATCCACT AGTTCTAGAG GATCCAGACA TGATAAGATA | 3600 |
| | CATTGATGAG TTTGGACAAA CCACAACTAG AATGCAGTGA AAAAAATGCT TTATTTGTGA | 3660 |
| | AATTGTGTGAT GCTATTGCTT TATTGTAAAC CATTATAAGC TGCAATAAAC AAGTTAACAA | 3720 |
| | CAACAATTGC ATTCAATTAA TGTTTCAGGT TCAGGGGGAG GTGTGGGAGG TTTTTAAAG | 3780 |
| 20 | CAAGTAAAC CTCTACAAAT GTGGTATGGC TGATTATGAT CCTGCAAGCC TCGTCGTCTG | 3840 |
| | GCCGGACCAC GCTATCTGTG CAAGGTCCCC GGACGCGCGC TCCATGAGCA GAGCGCCCGC | 3900 |
| | CGCCGAGGCA AGACTCGGGC GGCGCCCTGC CCGTCCCACC AGGTCAACAG GCGGTAACCG | 3960 |
| | GCCTCTTCAT CGGGAATGCG CGCGACCTTC AGCATCGCCG GCATGTCCCC TGGCGGACGG | 4020 |
| | GAAGTATCAG CTCGACCAAG CTTGGCGAGA TTTTCAGGAG CTAAGGAAGC TAAAATGGAG | 4080 |
| 25 | AAAAAAATCA CTGGATATAC CACCGTTGAT ATATCCAAT GGCATCGTAA AGAACATTTT | 4140 |
| | GAGGCATTTC AGTCAGTTGC TCAATGTACC TATAACCAGA CCGTTCAGCT GCATTAATGA | 4200 |
| | ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGC GCTCTTCCGC TTCTCGCTC | 4260 |
| | ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGCGAGCGG TATCAGCTCA CTCAAAGGCG | 4320 |
| | GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC | 4380 |
| 30 | CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTCCA TAGGCTCCGC | 4440 |

- 57 -

| | | |
|----|---|------|
| | CCCCCTGACG ACCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA | 4500 |
| | CTATAAAGAT ACCAGGCAGT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC | 4560 |
| | CTGCCGCTTA CCGGATAACCT GTCCGCCCTT CTCCCTCGG GAAGCGTGGC GCTTTCTCAA | 4620 |
| | TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTG GCTCCAAGCT GGGCTGTGTG | 4680 |
| 5 | CACGAACCCC CGGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC | 4740 |
| | AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA | 4800 |
| | GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCTTAACTA CGGCTACACT | 4860 |
| | AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTCGG AAAAAGAGTT | 4920 |
| | GGTAGCTCTT GATCCGGCAA ACAAACCAACC GCTGGTAGCG GTGGTTTTT TGTTGCAAG | 4980 |
| 10 | CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG | 5040 |
| | TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATT TGTCATGAG ATTATCAAAA | 5100 |
| | AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT TAAATCAAT CTAAAGTATA | 5160 |
| | TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG | 5220 |
| | ATCTGTCTAT TTCGTTCATC CATAGTTGCC TGACTCCCCG TCCTGTAGAT AACTACGATA | 5280 |
| 15 | CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG | 5340 |
| | GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT | 5400 |
| | GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAGCTAG AGTAAGTAGT | 5460 |
| | TCGCCAGTTA ATAGTTGCG CAACGTTGTT GCCATTGCTA CAGGCATCGT GGTGTCACCG | 5520 |
| | TCGTCGTTG GTATGGCTTC ATTCAAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA | 5580 |
| 20 | TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAACT | 5640 |
| | AAGTTGGCCG CAGTGTATC ACTCATGGTT ATGGCAGCAC TGCTATAATTG TCTTACTGTC | 5700 |
| | ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC ATTCTGAGAA | 5760 |
| | TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGCGTCAA TACGGGATAA TACCGCGCCA | 5820 |
| | CATAGCAGAA CTTTAAAGT GCTCATCATT GGAAACGTT CTTCGGGGCG AAAACTCTCA | 5880 |
| 25 | AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCTGTCACC CAACTGATCT | 5940 |
| | TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC | 6000 |
| | GCAAAAAAGG GAATAAGGGC GACACGGAAA TGTTGAATAC TCATACTCTT CCTTTTTCAA | 6060 |
| | TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT | 6120 |
| | TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGTC | 6180 |
| 30 | TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCCTATCAC GAGGCCCTTT | 6240 |

CGTC

6244

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 4963 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

10 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Human cytomegalovirus

(vii) IMMEDIATE SOURCE:
 (B) CLONE: pUHD BGR4

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

| | |
|---|------|
| CTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT TACCACTCCC | 60 |
| TATCAGTGAT AGAGAAAAGT GAAAGTCGAG TTTACCACTC CCTATCAGTG ATAGAGAAAA | 120 |
| GTGAAAGTCG AGTTTACAC TCCCTATCG TGATAGAGAA AAGTGAAAGT CGAGTTTACC | 180 |
| ACTCCCTATC AGTGATAGAG AAAAGTGAAG GTCGAGTTA CCACTCCCTA TCAGTGATAG | 240 |
| 20 AGAAAAGTGA AAGTCGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG | 300 |
| CTCGGTACCC GGGTCGAGTA GGCGTGTACG GTGGGAGGCC TATATAAGCA GAGCTCGTTT | 360 |
| AGTGAACCGT CAGATCGCCT GGAGACGCCA TCCACGCTGT TTTGACCTCC ATAGAACGACA | 420 |
| CCGGGACCGA TCCAGCCTCC GCGGCCCCGA ATTCCGGCCA CGACCATGAC CATGACCCCTC | 480 |
| CACACCAAAG CATCTGGGAT GGCCTACTG CATCAGATCC AAGGGAACGGA GCTGGAGCCC | 540 |
| 25 CTGAACCGTC CGCAGCTCAA GATCCCCCTG GAGCGGCCCG TGGGCGAGGT GTACCTGGAC | 600 |
| AGCAGCAAGC CCGCCGTGTA CAACTACCCC GAGGGCGCCG CCTACGGAGTT CAACGCCCG | 660 |
| GCCGCCGCCA ACGCGCAGGT CTACGGTCAG ACCGGCCTCC CCTACGGCCC CGGGTCTGAG | 720 |
| GCTGCGGCCTG TCGGCTCCAA CGGCCTGGGG GGTTCCCCC CACTAACAG CGTGTCTCCG | 780 |
| AGCCCGCTGA TGCTACTGCA CCCGCCGCCG CAGCTGTCGC CTTTCCTGCA GCCCCACGGC | 840 |
| 30 CAGCAGGTGC CCTACTACCT GGAGAACGAG CCCAGCGGCT ACACGGTGCG CGAGGCCGGC | 900 |
| CCGCCGGCAT TCTACAGGCC AAATTCAAGAT AATCGACGCC AGGGTGGCAG AGAAAGATTG | 960 |
| GCCAGTACCA ATGACAAGGG AAGTATGGCT ATGGAATCTG CCAAGGAGAC TCGCTACTGT | 1020 |
| GCAGTGTGCA ATGACTATGC TTCAGGCTAC CATTATGGAG TCTGGTCCTG TGAGGGCTGC | 1080 |
| AAGGCCTTCT TCAAGAGAAG TATTCAAGGA CATAACGACT ATATGTGTCC AGCCACCAAC | 1140 |
| 35 CAGTGCACCA TTGATAAAAAA CAGGAGGAAG AGCTGCCAGG CCTGCCGGCT CCGCAAATGC | 1200 |

- 59 -

| | | | | | | | |
|----|-------------|------------|-------------|-------------|------------|-------------|------|
| | TACGAAGTGG | GAATGATGAA | AGGTGGGATA | CGAAAAGACC | GAAGAGGAGG | GAGAATGTTG | 1260 |
| | AAACACAAGC | GCCAGAGAGA | TGATGGGGAG | GGCAGGGGTG | AAGTGGGGTC | TGCTGGAGAC | 1320 |
| | ATGAGAGCTG | CCAACCTTTG | GCCAAGCCCG | CTCATGATCA | AACGCTCTAA | GAAGAACAGC | 1380 |
| | CTGGCCTTGT | CCCTGACGGC | CGACCAGATG | GTCATGCCCT | TGTTGGATGC | TGAGCCCCCC | 1440 |
| 5 | ATACTCTATT | CCGAGTATGA | TCCTACCAGA | CCCTTCAGTG | AAGCTTCGAT | GATGGGCTTA | 1500 |
| | CTGACCAACC | TGGCAGACAG | GGAGCTGGTT | CACATGATCA | ACTGGGCGAA | GAGGGTAGCCA | 1560 |
| | GGCTTGTGG | ATTTGACCT | CCATGATCAG | GTCCACCTTC | TAGAATGTGC | CTGGCTAGAG | 1620 |
| | ATCCTGATGA | TTGGTCTCGT | CTGGCGCTCC | ATGGAGCACC | CAGTGAAGCT | ACTGTTGCT | 1680 |
| | CCTAACATTGC | TCTTGGACAG | GAACCAGGGA | AAATGTGTAG | AGGGCATGGT | GGAGATCTTC | 1740 |
| 10 | GACATGCTGC | TGGCTACATC | ATCTCGGTT | CGCATGATGA | ATCTGCAGGG | AGAGGAGTTT | 1800 |
| | GTGTGCCTCA | AATCTATTAT | TTTGCTTAAT | TCTGGAGTGT | ACACATTCT | GTCCAGCACC | 1860 |
| | CTGAAGTCTC | TGGAAGAGAA | GGACCATATC | CACCGAGTCC | TGGACAAGAT | CACAGACACT | 1920 |
| | TTGATCCACC | TGATGGCAA | GGCAGGCCTG | ACCCTGCAGC | AGCAGCACCA | GCGGCTGGCC | 1980 |
| | CAGCTCCTCC | TCATCCTCTC | CCACATCAGG | CACATGAGTA | ACAAAGGCAT | GGAGCATCTG | 2040 |
| 15 | TACAGCATGA | AGTGAAGAA | CGTGGTGC | CTCTATGACC | TGCTGCTGG | GATGCTGGAC | 2100 |
| | GCCCACCGCC | TACATGCGCC | CACTAGCCGT | GGAGGGGCAT | CCGTGGAGGA | GACGGACCAA | 2160 |
| | AGCCACTTGG | CCACTGCGGG | CTCTACTTCA | TCGCATTCC | TGCAAAAGTA | TTACATCACG | 2220 |
| | GGGGAGGCAG | AGGGTTTCCC | TGCCACAGTC | TGAGAGCTCC | CTGGCGGAAT | TCGAGCTCGG | 2280 |
| | TACCCGGGGA | TCCTCTAGAG | GATCCAGACA | TGATAAGATA | CATTGATGAG | TTTGGACAAA | 2340 |
| 20 | CCACAACTAG | AATGCAGTGA | AAAAAATGCT | TTATTTGTGA | AATTTGTGAT | GCTATTGCTT | 2400 |
| | TATTTGTAAC | CATTATAAGC | TGCAATAAAC | AAGTTAACAA | CAACAATTGC | ATTCAATTAA | 2460 |
| | TGTTTCAGGT | TCAGGGGGAG | GTGTGGGAGG | TTTTTAAAG | CAAGTAAAC | CTCTACAAAT | 2520 |
| | GTGGTATGGC | TGATTATGAT | CCTGCAAGCC | TCGTCGTCTG | GCCGGACCAC | GCTATCTGTG | 2580 |
| | CAAGGTCCCC | GGACGCGCGC | TCCATGAGCA | GAGCGCCCGC | CGCCGAGGCA | AGACTCGGGC | 2640 |
| 25 | GGGCCCTGC | CCGTCCCACC | AGGTCAACAG | GCGGTAACCG | GCCTCTTCAT | CGGGATGCG | 2700 |
| | CGCGACCTTC | AGCATGCCG | GCATGTCCCC | TGGCGGACGG | GAAGTATCAG | CTCGACCAAG | 2760 |
| | CTTGGCGAGA | TTTCAGGAG | CTAAGGAAGC | TAAAATGGAG | AAAAAAATCA | CTGGATATAC | 2820 |
| | CACCGTTGAT | ATATCCCAAT | GGCATCGTAA | AGAACATTTC | GAGGCATTTC | AGTCAGTTC | 2880 |
| | TCAATGTACC | TATAACCAGA | CCGTTCAAGCT | GCATTAATGA | ATCGGCCAAC | GCGCGGGGAG | 2940 |
| 30 | AGGCGGTTTG | CGTATTGGC | GCTCTCCGC | TTCCCTCGCTC | ACTGACTCGC | TGCGCTCGGT | 3000 |

- 60 -

| | | |
|----|---|------|
| | CGTTGGGCTG CGGCAGCGG TATCAGCTCA CTCAAAGGCG GTAATACGGT TATCCACAGA | 3060 |
| | ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG | 3120 |
| | TAAAAAGGCC GCGTTGCTGG CGTTTTCCA TAGGCTCCGC CCCCCTGACG AGCATCACAA | 3180 |
| | AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGC GTT | 3240 |
| 5 | TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC CTGCCGCTTA CCGGATAACCT | 3300 |
| | GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAA TGCTCACGCT GTAGGTATCT | 3360 |
| | CAGTTCGGTG TAGGTGTTG GCTCCAAGCT GGGCTGTGTG CACGAACCCC CCGTTCAGCC | 3420 |
| | CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA GACACGACTT | 3480 |
| | ATCGCCACTG GCAGCAGCCA CTGGTAAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTG | 3540 |
| 10 | TACAGAGTTC TTGAAGTGGT GGCTAACTA CGGCTACACT AGAAGGACAG TATTTGGTAT | 3600 |
| | CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA | 3660 |
| | ACAAAACCAC C GCTGGTAGCG GTGGTTTTT TGGTTGCAAG CAGCAGATTA CGCGCAGAAA | 3720 |
| | AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC AGTGGAAAGCA | 3780 |
| | AAAACTCACGT TAAGGGATTG TGGTCATGAG ATTATCAAAA AGGATCTTCA CCTAGATCCT | 3840 |
| 15 | TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA TATGAGTAAA CTTGGTCTGA | 3900 |
| | CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT TTCGTTCATC | 3960 |
| | CATAGTTGCC TGATCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC | 4020 |
| | CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTG ATCAGCAATA | 4080 |
| | AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGCCTG CAACTTTATC CGCCTCCATC | 4140 |
| 20 | CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTGCGC | 4200 |
| | AACGTTGTTG CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTGG TATGGCTTCA | 4260 |
| | TTCAAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAAA | 4320 |
| | GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTATCA | 4380 |
| | CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT | 4440 |
| 25 | TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATCGC GCGACCGAGT | 4500 |
| | TGCTCTTGCC CGGGCGTCAAT ACGGGATAAT ACCCGGCCAC ATAGCAGAAC TTTAAAAGTG | 4560 |
| | CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACCGCTGTTGAGA | 4620 |
| | TCCAGTTCGA TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTT TACTTTCAC | 4680 |
| | AGCGTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG | 4740 |
| 30 | ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTCAAT ATTATTGAAG CATTATCAG | 4800 |

- 61 -

| | |
|--|------|
| GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAAATAGGG | 4860 |
| GTTCCCGCGCA CATTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG | 4920 |
| ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTC GTC | 4963 |

5 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGAGTTTAC CACTCCCTAT CAGTGATAGA GAAAAGTGAA AG

42

CLAIMS

1. An isolated DNA molecule for integrating a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) at a predetermined location in a second target DNA molecule, the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells, the DNA molecule comprising a polynucleotide sequence encoding the tTA flanked at 5' and 3' ends by additional polynucleotide sequences of sufficient length for homologous recombination between the DNA molecule and the second target DNA molecule at a predetermined location.
10
2. The DNA molecule of claim 1, wherein the additional polynucleotide sequences flanking the polynucleotide sequence encoding the tTA are of a gene of interest, or regulatory region thereof, into which the DNA molecule is inserted.
- 15 3. The DNA molecule of claim 2, wherein, upon integration of the DNA molecule into the gene of interest, or regulatory region thereof, expression of the tTA is controlled by regulatory elements of the gene of interest.
- 20 4. The DNA molecule of claim 1, wherein the Tet repressor of the tTA is a Tn10-derived Tet repressor.
- 25 5. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is from herpes simplex virus virion protein 16.
- 30 6. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is selected from the group consisting of acidic, proline-rich, serine/threonine-rich and glutamine-rich transcriptional activation polypeptides.
- 35 7. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain selected from the group consisting of a leucine zipper domain, a helix-loop-helix domain and a zinc finger domain.
8. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain from a TATA binding protein.

9. The DNA molecule of claim 1, further comprising a polynucleotide sequence encoding a selectable marker.

10. The DNA molecule of claim 9, wherein the nucleotide sequence encoding the 5 selectable marker is a tk gene or a neomycin resistance gene.

11. An isolated DNA molecule for integrating a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) and a tTA-responsive promoter within a predetermined gene of interest in a second target DNA molecule, the DNA molecule 10 comprising:

- a) a first polynucleotide sequence comprising a 5' flanking regulatory region of the gene of interest, operably linked to;
- b) a second polynucleotide sequence encoding a tTA, the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells; and
- c) a third polynucleotide sequence comprising a tTA-responsive promoter, operably linked to;
- d) a fourth polynucleotide sequence comprising at least a portion of a coding 20 region of the gene of interest;

wherein the first and fourth polynucleotide sequences are of sufficient length for homologous recombination between the DNA molecule and the gene of interest in the second target DNA molecule such that expression of the tTA is controlled by 5' regulatory elements of the gene 25 of interest and expression of the gene of interest is controlled by the tTA-responsive promoter.

12. The DNA molecule of claim 11, further comprising a fifth polynucleotide sequence encoding a selectable marker operably linked to a regulatory sequence, wherein the fifth 30 polynucleotide sequence is located between the second and third polynucleotide sequences.

13. The DNA molecule of claim 12, wherein the fifth polynucleotide sequence encoding the selectable marker is a tk gene or a neomycin resistance gene.

35 14. The DNA molecule of claim 11, further comprising a fifth polynucleotide sequence comprising a transcriptional terminator signal, a transcriptional insulator or a matrix attachment region, wherein the fifth polynucleotide sequence is located between the second and third polynucleotide sequences.

15. The DNA molecule of claim 12, further comprising a sixth polynucleotide sequence comprising a transcriptional terminator signal, a transcriptional insulator or a matrix attachment region, wherein the sixth polynucleotide sequence is located between the fifth and third polynucleotide sequences.

5

16. The DNA molecule of claim 11, wherein the Tet repressor of the tTA is a Tn10-derived Tet repressor.

10 17. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is from herpes simplex virus virion protein 16.

15 18. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is selected from the group consisting of acidic, proline-rich, serine/threonine-rich and glutamine-rich transcriptional activation polypeptides.

20 19. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain selected from the group consisting of a leucine zipper domain, a helix-loop-helix domain and a zinc finger domain.

25 20. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain from a TATA binding protein.

21. The DNA molecule of claim 11, wherein the tTA-responsive promoter of the third nucleotide sequence comprises a minimal promoter operably linked to at least one tet operator sequence.

30

22. The DNA molecule of claim 21, wherein the minimal promoter is derived from a cytomegalovirus immediate early gene promoter or a herpes simplex virus thymidine kinase gene promoter.

35 23. The DNA molecule of claim 11, wherein the gene of interest is a human gene.

24. The DNA molecule of claim 23, wherein the human gene encodes a gene product selected from the group consisting of adenosine deaminase, Factor VIII, Factor IX, dystrophin, β-globin, LDL receptor, CFTR, insulin, erythropoietin, anti-angiogenesis factors,

- 65 -

growth hormone, glucocerebrosidase, β -glucuronidase, $\alpha 1$ -antitrypsin, phenylalanine hydroxylase, tyrosine hydroxylase, ornithine transcarbamylase, arginosuccinate synthetase, UDP-glucuronyl transferase, apoA1, MDR1, MRP, TNF, soluble TNF receptor, an interleukins, an interferon, a cytokine, a growth factor and a tumor suppressor gene.

5 25. A eucaryotic host cell comprising the DNA molecule of claim 1, wherein the DNA molecule is integrated at a predetermined location in a second target DNA molecule in the host cell.

10 26. The host cell of claim 25, further comprising a gene of interest operably linked to a tTA-responsive transcriptional promoter.

27. The host cell of claim 26, wherein the tTA-responsive promoter comprises a minimal promoter operably linked to at least one tet operator sequence.

15 28. The host cell of claim 27, wherein the minimal promoter is derived from a cytomegalovirus immediate early gene promoter or a herpes simplex virus thymidine kinase gene promoter.

20 29. The host cell of claim 25, which is a mammalian cell.

30. The host cell of claim 29, which is a human cell.

31. The host cell of claim 29, which is an embryonic stem cell.

25 32. The host cell of claim 25, which is a yeast cell or a fungal cell.

33. The host cell of claim 25, wherein the cell is an insect cell and the second target DNA molecule is an insect gene or a baculovirus gene.

30 34. A method for inhibiting transcription of the gene of interest operatively linked to the tTA-responsive promoter in the host cell of claim 26, comprising contacting the cell with tetracycline or a tetracycline analogue.

35 35. A host cell comprising the nucleic acid of claim 11, wherein the nucleic acid is integrated into the predetermined gene of interest in a second target DNA molecule in the host cell.

36. The host cell of claim 35, which is a mammalian cell.

37. The host cell of claim 36, which is a human cell.
38. The host cell of claim 36, which is an embryonic stem cell.
5
39. The host cell of claim 35, which is a yeast cell or a fungal cell.
40. The host cell of claim 35, wherein the cell is an insect cell and the gene of interest is an insect gene or a baculovirus gene.
10
41. A method for inhibiting transcription of the eucaryotic gene of interest in the host cell of claim 35, comprising contacting the cell with tetracycline or a tetracycline analogue.
42. A non-human transgenic animal having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA), the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells.
15
43. The animal of claim 42, wherein the Tet repressor of the tTA is a Tn10-derived Tet repressor.
20
44. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is from herpes simplex virus virion protein 16.
25
45. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is selected from the group consisting of acidic, proline-rich, serine/threonine-rich and glutamine-rich transcriptional activation polypeptides.
30
46. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain selected from the group consisting of a leucine zipper domain, a helix-loop-helix domain and a zinc finger domain.
35
47. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain from a TATA binding protein.

48. The animal of claim 42, further having a second transgene comprising a gene of interest operably linked to a tTA-responsive promoter.

49. The animal of claim 42, which is a mouse.

5

50. The animal of claim 42, which is selected from a group consisting of a cow, a goat, a sheep and a pig.

51. A method for inhibiting transcription of the second transgene in the transgenic animal
10 of claim 48, comprising administering tetracycline or a tetracycline analogue to the animal.

52. A non-human transgenic animal having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA), the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly
15 activates transcription in eucaryotic cells, wherein the transgene is integrated by homologous recombination at a predetermined location within a chromosome within cells of the animal.

53. The animal of claim 52, further having a second transgene comprising a gene of interest operably linked to a tTA-responsive promoter.

20

54. A method for inhibiting transcription of the second transgene in the animal of claim 53, comprising administering tetracycline or a tetracycline analogue to the animal.

55. A transgenic animal having a transgene comprising a polynucleotide sequence
25 encoding a tetracycline-controllable transactivator (tTA) and a tTA-responsive promoter, wherein the transgene is integrated by homologous recombination at a predetermined location within a gene of interest within cells of the animal such that expression of the tTA is controlled by 5' regulatory elements of the gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter.

30

56. A method for inhibiting transcription of the gene of interest in the animal of claim 55, comprising administering tetracycline or a tetracycline analogue to the animal.

57. A process for producing and isolating a gene product encoded by the gene of interest
35 operably linked to the tTA-responsive transcriptional promoter in the cell of claim 26, comprising:

- a) growing cells in a culture medium in the presence of tetracycline or a tetracycline analogue;
- b) reducing the concentration of tetracycline or the tetracycline analogue to stimulate

transcription of the gene of interest;

- c) further culturing the cells until a desired amount of the gene product encoded by the gene of interest is produced by the cells; and
- b) isolating the gene product from harvested cells or from the culture medium.

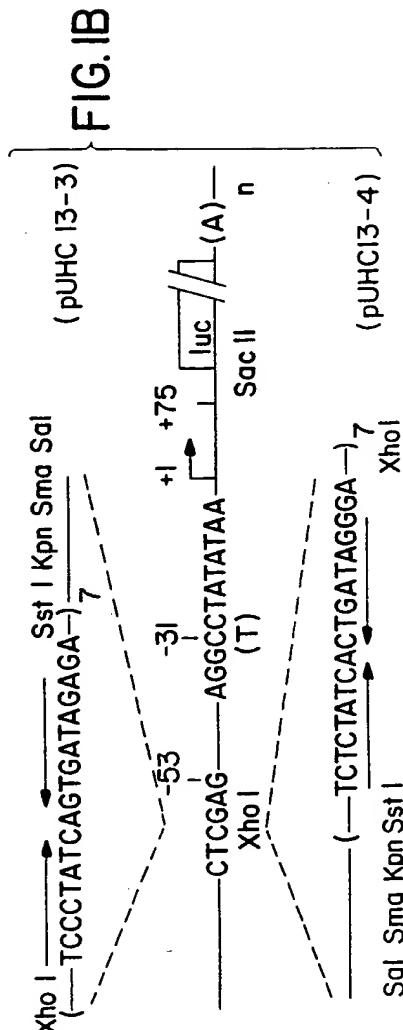
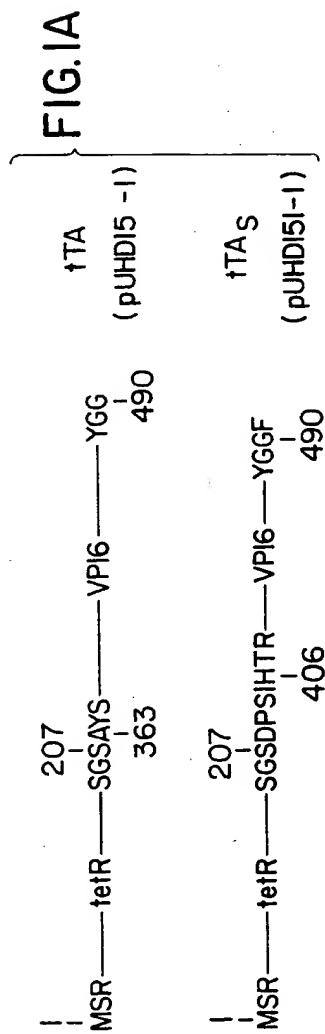
5

- 58. The process of claim 57, wherein the cells are mammalian cells.
- 59. The process of claim 57, wherein the cells are yeast or fungal cells.
- 10 60. A method for producing the non-human transgenic animal of claim 42, comprising:
 - a) introducing a DNA molecule encoding the tTA into a fertilized oocyte;
 - b) implanting the fertilized oocyte in a pseudopregnant foster mother; and
 - c) allowing the fertilized oocyte to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal.
- 15 61. A method for producing a host cell having a DNA molecule encoding a tetracycline-controllable transactivator (tTA) integrated at a predetermined location in a second target DNA molecule within the cell, comprising:
 - a) introducing the DNA molecule of claim 1 into a population of cells under conditions suitable for homologous recombination between the DNA encoding the tTA and the second target DNA molecule; and
 - b) selecting a cell in which the DNA encoding the tTA has integrated at a predetermined location within the second target DNA molecule.
- 20 62. A method for producing a non-human transgenic animal having a transgene encoding a tetracycline-controllable transactivator (tTA) integrated at a predetermined location within chromosomal DNA of cells of the animal, comprising:
 - a) introducing the DNA molecule of claim 1 into a population of embryonic stem cells under conditions suitable for homologous recombination between the DNA encoding the tTA and chromosomal DNA within the cell;
 - b) selecting an embryonic stem cell in which DNA encoding the tTA has integrated at a predetermined location within the chromosomal DNA of the cell;
 - c) implanting the embryonic stem cell into a blastocyst;
 - d) implanting the blastocyst into a pseudopregnant foster mother; and
 - 30 e) allowing the blastocyst to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal.
- 35 63. A method for producing a non-human transgenic animal having a transgene encoding a tetracycline-controllable transactivator (tTA) and a tTA-responsive promoter integrated at a

predetermined location within a gene of interest in cells of the animal, comprising:

- a) introducing the DNA molecule of claim 11 into a population of embryonic stem cells under conditions suitable for homologous recombination between the DNA encoding the tTA and the gene of interest within the cell;
- 5 b) selecting an embryonic stem cell in which DNA encoding the tTA has integrated at a predetermined location within the gene of interest in the cell;
- c) implanting the embryonic stem cell into a blastocyst;
- d) implanting the blastocyst into a pseudopregnant foster mother; and
- e) allowing the blastocyst to develop into the non-human transgenic animal to thereby
10 produce the non-human transgenic animal.

1/32



SUBSTITUTE SHEET (RULE 26)

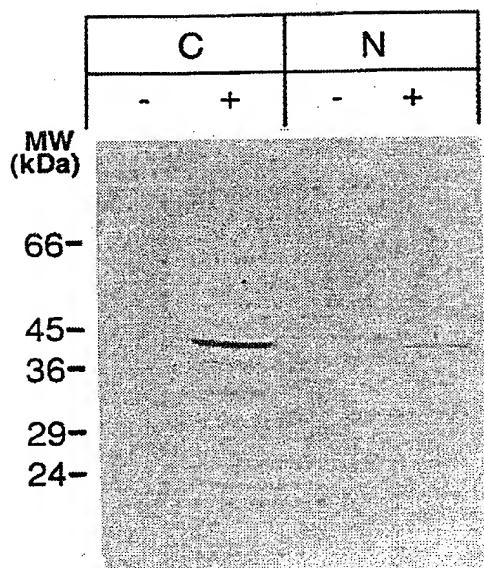


FIG. 2A

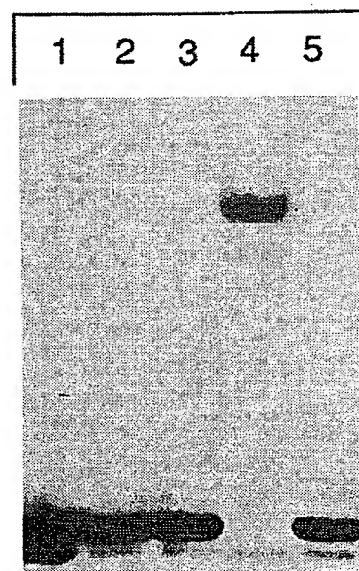


FIG. 2B

SUBSTITUTE SHEET (RULE 26)

3/32

FIG. 3B

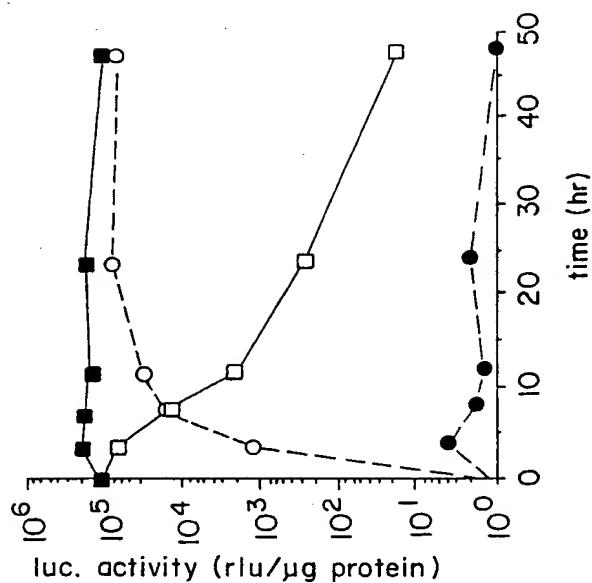
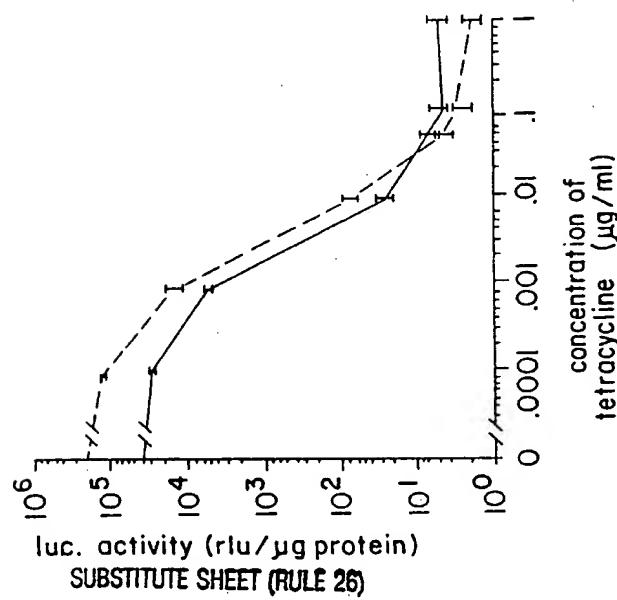


FIG. 3A



SUBSTITUTE SHEET (RULE 26)

ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG CTT AAT
 Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu Leu Asn

GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA
 Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln Lys Leu Gly Val

GAG CAG CCT ACA TTG TAT TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC
 Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys Arg Ala Leu Leu Asp Ala

TTA GCC ATT GAG ATG TTA GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG
 Leu Ala Ile Glu Met Leu Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly

GAA AGC TGG CAA GAT TTT TTA CGT AAT AAG GCT AAA AGT TTT AGA TGT GCT TTA
 Glu Ser Trp Gln Asp Phe Leu Arg Asn Lys Ala Lys Ser Phe Arg Cys Ala Leu

SUBSTITUTE SHEET (RULE 26)

Fig. 4A

CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA GAA AAA
Leu Ser His Arg Asp Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys

CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TRT TTA TGC CAA CAA GGT TTT TCA
Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser

CTA GAG AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT ACT TTA GGT TGC
Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys

GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA CCT ACT
Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr Pro Thr

ACT GAT AGT ATG CCG CCA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA
Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu Phe Asp His Gln

SUBSTITUTE SHEET (RULE 26)

Fig. 4B

GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA
Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu Ile Ile Cys Gly Leu Glu

AAA CAA CTT AAA TGT GAA AGT GGG TCC GCG TAC AGC CGC GCG CGT ACG AAA AAC
Lys Gln Leu Lys Cys Glu Ser Gly Ser Ala Tyr Ser Arg Ala Arg Thr Lys Asn

AAT TAC GGG TCT ACC ATC GAG GGC CTG CTC GAT CTC CCG GAC GAC GAC GCC CCC
Asn Tyr Gly Ser Thr Ile Glu Gly Leu Leu Asp Leu Pro Asp Asp Asp Ala Pro

GAA GAG GCG GGG CTG GCG GCT CCG CGC CTG TCC TTT CTC CCC GCG GGA CAC ACG
Glu Glu Ala Gly Leu Ala Ala Pro Arg Leu Ser Phe Leu Pro Ala Gly His Thr

CGC AGA CTG TCG ACG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC
Arg Arg Leu Ser Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His

SUBSTITUTE SHEET (RULE 26)

Fig. 4C

TAA GAC GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT
Leu Asp Gly Glu Asp Val Ala His Ala Asp Ala Leu Asp Asp Phe Asp

CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC CAC GAC
Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Pro Thr Pro His Asp

TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG CAG ATG ATG TTT
Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe

ACC GAT CCC CTT GGA ATT GAC GAG TAC GGT GGG TAG
Thr Asp Pro Leu Gly Ile Asp Glu Tyr Gly Gly *

SUBSTITUTE SHEET (RULE 26)

Fig. 4D

ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG CTT AAT
Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu Leu Asn

GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA
Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln Lys Leu Gly Val

GAG CAG CCT ACA TTG TAT TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC
Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys Arg Ala Leu Leu Asp Ala

TTA GCC ATT GAG ATG TTA GAT AGG CAC CAT ACT CAC TTT TGCG CCT TTA GAA GGG
Leu Ala Ile Glu Met Leu Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly

GAA AGC TGG CAA GAT TTT TTA CGT AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA
Glu Ser Trp Gln Asp Phe Leu Arg Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu

CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA GAA AAA
Leu Ser His Arg Asp Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys

CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA TGC CAA CAA GGT TTT TCA
Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser

CTA GAG AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT ACT TTA GGT TGC
Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu GLY Cys

GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA CCT ACT
Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr Pro Thr

ACT GAT AGT ATG CCG CCA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA
Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu Phe Asp His Gln

SUBSTITUTE SHEET (RULE 26)

Fig. 5B

GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA
 Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu Ile Ile Cys Gly Leu Glu

AAA CAA CTT AAA TGT GAA AGT GGG TCT GAT CCA TCG ATA CAC ACG CGC AGA CTG
 Lys Gln Leu Lys Cys Glu Ser Gly Ser Asp Pro Ser Ile His Thr Arg Arg Leu

TCG ACG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC
 Ser Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly

GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG
 Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met

TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC CAC GAC TCC GCC CCC
 Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro

SUBSTITUTE SHEET (RULE 26)

Fig. 5C

TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG CAG CTT ACC GAT GCC
Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Glu Gln Met Phe Thr Asp Ala

CTT GGA ATT GAC GAG TAC GGT GGG TTC TAG
Leu Gly Ile Asp Glu Tyr Gly Gly Phe *

Fig 5D

GAATTCCCTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTC
CCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGT
GAAAGTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTCCC
TATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGA
AAGTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGCTCGGTACCCGGGT
CGAGTAGGCCTACGGTGGAGGCCATATAAGCAGAGCTCGTTTAGTGAACCGTCAAGATCGC
CTGGAGACGCCATCCACGGCTGGTTGACCTCCATAGAAGACACCGGACCCGATCCAGCCTCCGC
GG

Fig. 6

SUBSTITUTE SHEET (RULE 26)

GAATTCTCGACCCGGGTACCGAGCTCGACTTTCACTTTTCTACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTA
AACTCGACTTTCACTTTCTACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTAA
ATCACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTAA
CTCGACTTTCACTTTCTACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTAA
CACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTAAACTCGACCTTCACCTTTCTACTGATAAGGGAGTGGTAA
CGAGTAGGGGTGTACGGTGGAGGGCTATAAAGCAGAGCTCGTTAGTGAAACCGTCAGATCGC
CTGGAGACGCCATCCACGCTGTTGACCTCCATAGAAGACACCGGACCGATCCAGCCTCCGC
GG

Fig. 7

GAGCTCGACTTTCACTTCTATCACTGATAAGGGAGTGGTAAACTCGACTTTCACTTTCTCT
TATCACTGATAAGGGAGTGGTAAACTCGACTTTCACTTCTATCACTGATAAGGGAGTGGTAAAC
ACTCGACTTTCACTTCTATCACTGATAAGGGAGTGGTAAACTCGACTTTCACTTCTCT
TCAGTGGAGTGGTAAACTCGACTTTCACTTCTATCACTGATAAGGGAGTGGTAAAC
TCGACTTTCACTTCTATCACTGATAAGGGAGTGGTAAACTCGAGATCCGGCGAATTCGAAC
ACGCAGATGCAGTCGGGGGGGGGGTCCGAGGTCCACTTCGCAATTAAAGGTGACGGGTGTGG
CCTCGAACACCGAG

Fig. 8

Fig. 9A

SUBSTITUTE SHEET (RULE 26)

GCTCTTCCCCGGCCCTGTCAAGGGCAGAACCCCCAGACGGGAAAGACGGCAGGCCACCGCT
TTGTCAAGACGTGGAGGGCATTTCTGGAGTCAGGCCACTTGCTGGACAGTGTCAAGCCCCGGAGGGCAGGAGACAGCAGCT
CGAGACCTCCAGAAAAGGACAGCGGGCTCTGGCTGACACGCTCAGACACGCTCCTGGCCCTC
GGGTCCCCGGCAGAGCCACGCCAGCCCCGGCTGCCACTTGCGAGGCCATCAGCCCCGGCTGTGGCCCTC
GGCCCCGAGCCTTCCCGAAGAACCCCCGGCTGCCACTTGCGAGGCCATCAGCCCCGGCTGTGGCCCTC
TGAGCCGAGCCGAGCAAGGCAGGGCACAGCTCTGGAGCCAGGGCAGGGCACAGCTCAGGGAGCCCTCACTGGAGGCC
CAGGGGACTGTCAACCATCCCAAGGCCACTGCTGCTGCCCTCTCTGGAGGCC
GTGAAGGCCATCCCCGGAGCCCGCTGGCGTAGGCTGAGGCCAGGAGACAGCTCCGAATCCGAGG
GCACCGTGGCCCGCTCCTGAAGGGCCAACCTGGCAGCTGGGAGGGCACGGGGGGAGGGAGG
AGCTGCCCTCGCGTCTGGAGGGCCAGGGCGTGCCTGGGGAGCAGGAGCCCTGGGGCTGGGG
CGCTTCTCGGGCCCAGGGTCTCCCTGGGGAGCAGGAGCCCTGGGGCTGGGG
CGCTGCCACCTCGTGGATTCACTCACGTGCCATCCTGCTCTCAACCACGTTTCT
GGCCACCCGGACCAAGGCAAGCTGCTGGAGGCTAACCAAGGGAGGCTAACCGGGCCAGC

Fig. 9B

CCCTTGTCCCCGAGGGGCTCCCCCTCTCGTCCACCCCTGTGGGGGGGGGACTTTCC
CCGACTGACCTACCCGCCGGAGCCAAAGATGACGCCCTCTACGGGACTT
CCAGCCGCCGCCCTCAAGATAAAGGAGGAAGAACGCCGGAGGGCTCCCCCG
CGTACGTTACCTGGTGGCTGGCAAAACCCCAGCCTTCCCGGACTTCAGCTGGCAGGCCGC
CGCCACCCCTCGGTGGCTCGAGTGCCTCGTCCAGAACCCGGGAAGGCCGGTGGGGCCT
CCCAGGGCAGTGCTCCGTCTCCCTCGTCGGGTCGACCCCTGGAGTGCATCCTGTAC
AAGGCAGAAGGGGGCCGCCAGCAGGGCCCTTCGGCCGCTGCCCTGCAAGGCCCTGGGGCG
CGGGCCTGCTCCCGGGAGCGGCCCTCACCTCCGGCTCGGGCAGGCCGG
GGCCGCCCTGGCTCACCCGACGCTGGCCTCAACGGACTCCGGAACTCGGCTACCGGCC
GCCGTGCTCAAGGAGGGCTGGCGCAGGTCTACACGCCCTATCTCAACTACCTGAGGCCGGATT
CAGAAGGCCAGTCAGAGCCACAGTACAGCTGAGTCACTACCTCAGAAAGATTGTTGATCTG
TGGGGATGAAGCATCAGGCTGTCAATTATGGTGTCCCTCACCTGTGGAGCTGTAAGGTCTTCTTT
AAAAGGGCAATGGAAGGGCAGCATAACTATTATGTGCTGGAAGAAATGACTGCATTGTTGATA

Fig. 9C

AAATCCGGAGGAAAAACTGCCGGCGTGCCTTAGAAAAGTGCCTGAAGCTGGCATGGTC
TGGAGGGCGAAGTTAAAGTTCATAAAGTCAGAGTCATGAGAGCACTCGATGCTGTTGCT
CTCCCCACAGCCAGTGGCATTCCAATGAAAGCCAACGAATCACCTTTCTCCAAGTCAAGAGA
TACAGTTAATTCCCCCTCTTAATCAACCTGTAAATGAGCATTGAAACCAGATGTGATCTATGCAGG
ACATGACAACACAAAGCCCTGATAACCTCCCACTGCAGTCTTGCTGACGAGTCTTAATCAACTAGGCAGG
CGGCAACTTCTTCAGTGTAAATGGTCCAAATCTCTCCAGGTTTCGAAACTTACATATTG
ATGACCAAGATAACTCTCATCCAGTATTCTGGATGAGTTAACGGTTATTGGACTAGGGATGGAG
ATCCTACAAACATGTCAGTGGCAGATGCTGTATTGACCTGCTAAATTTAACAG
CGGATGAAAGAATCATCATCTTACCATGCCTTACCATGTCAGATACCGCAGGAGTTG
TCAAGCTTCAAGTTAGCCAAGAAGAGTTCCCTCTGCATGAAAGTTACTACTTCTTAATACAAT
TCCTTTGGAAGGACTAAGGAAGTCAAAGCCAGTTGAAGAGATGAGATCAAGCTACATTAGAGAG
CTCATCAAGGCAATTGGTTGAGGCAAAAGGAGTTCCAGCTCACAGCGTTCTATCAGC
TCACAAAACCTCTTGATGATACTTGATCTGTCAAACAACTTACCTGTACTGCTGAATAAC

Fig. 9D

ATTATCCAGTCCCCGGCTGAGTGTGAATTCCAGAAATGATGTTCTGAAAGTTATTGCTGCA
 CAGTTACCCAAAGATAATTGGCAGGGATGGTGAACCACTTCCTTCAATAAAAAGTGAAATGTCAA
 TTATTTCAAGAATTAAAGTGTGGTATGTCCTCGTTTGGTCAGGATTATGACGTCCTCG
 AGTTTTATAATATTCTGAAAGGGAAATTCCCTGCAGCCCCGGGATCCACTAGTTCTAGAGGATC
 CAGACATGATAAGATAACATTGATGAGTTGGACAACACAACTAGAACATGCACTGAAAAAAATG
 CTTTATTTGTGAAATTGTGATGCTATTGCTTATTGTAACCATTATAAGTGCATAAAACAA
 GTTAACAAACAAATTGCAATTCAATTGTTATGTTTCAAGGTTCAAGGGGAGGGTGTGGGAGGTTTTT
 AAAGCAAGTAAACCTCTACAAATGTTGATATTGCTGATTATGATCCTGCAAGCCTCGTCGTCTG
 GCGGACACGGCTATCTGTGCAAGGTCCCCGGACGGGGCTCCCATGAGCAGGGCCCTCTTC
 GAGGCAAGACTCGGGGGGCCCTGCCGTCCCACCAAGGGTCAACAGGGGTAAACAGGGCT
 ATCGGGGAATGCGGGGACCTTCAGCATTGCCATGTCGGGACGGGAAGGTATCAGCT
 CGACCAAGCTTGGCGAGATTTCAGGAGCTAAGGAAGCTAAATGGAGAAAAAATCACTGGAT
 ATACCAACCGTTGATATCCCAATGGCATCGTAAGGAAGCTTCAAGGCATTTCAGTCAGTTGC

Fig. 9E

TCAATGTACCTATAACCAGCCGTCAGCTGCATTAATGAATCGGCCAACGGGGAGAGGC
GGTTTGGTATTGGGGCTCTTCCGCTTCGCTCAGCTGACTGGCTGCCGCTCGGTCGGTTGGC
TGGGGAGGGTATCAGCTCACTCAAAGGGTAATAACGGTTATCCACAGAAATCAGGGATAA
CGCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTG
CTGGCCTTTCCATAGGTCAGGCCCCCTGACGAGCATCACAAAAATCCAGCCTCAAGTCAGA
GGTGGCGAAACCCGACAGGACTATAAGATAACCAGGGTTTCCCCCTGGAAAGCTCCCTCGTGCG
CTCTCCTGTTCCGACCCCTGCCGCTTACCGGATAACCTGTCGCCCTTCTCCCTCGGAAGCGTG
GGCCTTCTCAATGCTCACGCTGTAAGGTATCTCAGTTGGCTGTAAGGTCTGCTCCAAGCTGG
GCTGTTGCAAGAACCCCCGGTTCAAGCCGACCCGCTGCCCTATCCGGTAACTATGGTCTTGA
GTCCAACCCGGTAAGACACGACTTATGCCCACTGGCAGCCACTGGTAACAGGATTAGCAGA
GCGAGGGTATGTTGGGTGCTACAGAGTTCTGTAAGTGGGGCTAACTACGGCTACACTAGAA
GGACAGTATTGGTATCTGGCTCTGCTGAAGGGCCAGTTACCTTCGGAAAAGAGTTGGTAGCTC
TTGATCCGGCAAACACACCAGGCTGGTAGCCAGTTTGTGCAAGCAGATTACG

Fig. 9F

CGCAGAAAAAGGATCTCAAGAAGATCCTTGTATCTACGGGGTCTGACGGCTCAGTGGAA
 ACGAAAAGCTCAGTTAACGGGATTGGTCATGAGATTATCAAAGGATCTTCAACCTAGATCCCT
 TTTAATTAAATGAAGTTAACATCAATTAAAGTATATGAGTAACACTGGTCTGACAGT
 TACCAATGCTTAAATCAGTGAGGCACCTATCTAGCGATCTGGCTTACATCCATAGTTG
 CCTGACTCCCCCGTCGTGTAGATAACTACGGATAACGGGAGGGCTTACCATCTGGCCCCAGTGCTGC
 AATGATAACCGGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAACCAGGCCAGGGAA
 AGGGCCGAGCGAGAACGTCCTGCCAACATTATCCGGCTCCATCCAGTCTTAAATTGTTGCC
 GGGAAAGCTAGAGTAAGTAGTTGCCAGTTAAATAGTTGCCAACGGTTGCTGCAATTGCTACAGG
 CATCGTGGTGTCAACGCTCGTCGTTGGTATGGCTTCCAAAGGATCAAGG
 CGAGTTACATGATCCCCCATGTTGTGCAAAAAGGGTTAGCTCCTCCGATCGTTG
 TCAGAAAGTAAGTGGCCAGTGTATCACTCATGGTTATGGCTATGGCAGGACTGCATAATTCTCTTAC
 TGTCAATGCCATCGTAAGATGCTTCTGTGACTGGTGAGTACTCAACCAAGTCATCTGAGAA
 TAGTGTATGGCGACCGAGTTGCTCTGGCTCAATAACGGGATAATAACGGGCCACATA

Fig. 9G

GCAGAACTTTAAAAGTGAGATCCAGTTGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
ACCGCTGTGAGATCCAGTTGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
ACTTCACCAAGGGTTCTGGGTGAGCAAAACAGGAAGGCCAAATGCCGCAAAAAGGGAAATAA
GGGCACACGGAAATGTTGAATACTCATACTCTCCTTTCAAAATTATTGAAGCATTATCA
GGGTTATTGGTCTCATGAGGGATAACATATTGAATGTTATTAGAAAAATAAACAAATAAGGGTT
CCGGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATGACATTAA
CCTATAAAAATAGGGTATCACGAGGCCCTTCGTC

Fig. 9H

CTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGT
AGT GATAGAGAAAAGTGAAGTCGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTCCCTATCAG
CGAGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTACCACTCCCTATCAGTGAGCTCGAGCTGGTACCCGGTGCAGTA
AGTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGCTGGTACCCGGTGCAGTA
GGCGTGTACGGTGGAGGCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAAGATGGCCTGGAG
ACGCCATCCACGCTGTTGACCTCATAGAAGACACGGGACCGATCCAGCCTCCGGCCCC
GAATTCCGGCCACGACCATGACCATGACCCCTCACACCAAAAGCATCTGGGATGGCCCTACTGCA
TCAGATCCAAGGAAACGAGCTGGAGCCCTGAACCGTCCGCAGCTCAAGATCCCCCTGGAGCG
CCCCTGGCGGAGGTGTACCTGGACACGGCAAGCCCCGGCGTGTACAACA
CCTACGAGTTCAACGCCGGCCGCGCCAAACGCCAGGTCTACGGTCAGACGGCCTCCCTA
CGGCCCCGGGTCTGAGGCTGGGCTCAACGGCCTGGGTTCAGCTGGGGTTCCCCCACTCAAC
AGCGTGTCTCCGAGCCCGCTGATGCTACTGCACCCGCCGGCAGCTGCGCTTTCCTGCAGC

Fig. 10A

CCCAAGGCCAGCAGGTGCCCTACTACCTGGAGAACGAGCCCCAGGGCTACACGGGTGGCGAGGC
CGGCCCGCCGGCATTCTACAGGCCAAATTAGATAATCGACGCCAGGGTGGCAGAGAAAGATG
GCCAGTACCAATGACAAGGGAAAGTATGGCTATGGAATCTGCCAAGGAGACTCGCTACTGTGAG
TGTGCAATGACTATGCTCAGGCTTACCATTTAGGAGTCTGGTCCCTGTGAGGGCTGCAAGGCCTT
CTTCAGAGAAGTATTCAAGGACATAACGACTATAACGACTATAACGACTATAACGACTATAACGACT
GATAAAAACAGGGAGGAAGAGCTGCCAGGCCCTGCCGGCTCCGGCAAATGCTACGAAGTGGGAATGA
TGAAAGGTGGATACGAAAAGACCCAAAGAGGGAGAAATGTTGAAACACAAAGGCCAGAGAGA
TGATGGGGAGGGCAGGGTGAAGTGGGTCTGGTGGAGACATGAGAGCTGCCAACCTTTGGCA
AGCCCGCTCATGATCAAACGCTCTAAGAACAGGCCCTGGCCTTGACGGGCCGACCGAGA
TGGTCATGGCCTTGTGGATGCTGAGCCCCCATACTCTATTCCGAGTATGATCCTACCAGACC
CTTCAGTGAAGCTTCGATGATGGGTTACTGACCAACTGGCAGACAGGGAGCTGGTCACATG
ATCAACTGGCGAAGAGGGTGCCAGGGCTTGTGGATTGACCCCTCCATGATCAGGTCCACCTC
TAGAATGTGGCTAGAGATCCTGATGATGGTCTCGTGGCTCCATGGAGCACCCAGT

Fig. 10B

25/32

GAAGCTACTGTTGCTCCTAACTTGCTCTGGACAGGAACCAAGGAAAATGTGTAGAGGGCATG
* GTGGAGATCTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGCAGGGAG
AGGAGTTTGTGCTCAAATCTATTATTGCTTAATTCTGGAGTGTACACATTCTGTCCAG
CACCTGAAGTCTGTGGAAAGAGAACCATATCCACCGAGTCCTGGACAAAGATCACAGACACT
TTGATCCACCTGATGGCCAAGGCCAGGCCTGACCCCTGCGACGCAGCACCAGGGCTGGCCAGC
TCCTCCATCCCACATCAGGCACATGAGTAACAAAGGCATGGAGCATCTGTACAGCAT
GAAGTGCAGAACGTTGGTGCCTCTATGACTGCTGGAGATGCTGGACGCCACCTGGCCACTA
CATGGCCCCACTAGCCGTTGGAGGGCATCCGGAGGGAGACGGGACAAAGCCACTTGGCCACTG
CGGGCTCTACTTCATGCATTCTTGCAAAAGTATTACATCACGGGGAGGCAGGGGTTCC
TGCCACAGTCTGAGAGCTCCCTGGGGAAATTGAGCTCGGTACCCGGGATCCTAGAGGATC
CAGACATGATAAGATAACATTGATGAGTTGGACAAACACAACATTAGAATGCACTGAAAAAAATG
CTTATTGTAATTGTGATGCTATTGCTTATTGTAACCATTATAAGCTGCAATAAAACAA
GTTAACAAACAAATTGCATTCAATTGTTTATGTTTCAGGGTCAAGGGGAGGTGTTGGGGTTTTT

Fig. 10C

SUBSTITUTE SHEET (RULE 26)

AAAGGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCCTGCAAGCCTCGTCGTCGTCTG
GCCGGACCACGGCTATCTGTGCAAGGGTCCCCGGACGGCGCTCCATGAGCAGAGGCCGCCGCC
GAGGCAGAAGACTCGGGGGGCCCTGCCCGTCCCACAGGTCAACAGGGGTAACAGGGCTCTC
ATCGGGGAATGCGCGGACCTTCAGCATCGCCGGCATGTCCTGGGGACGGGAAGTATCAGCT
CGACCAAGCTTGGCGAGATTTCAGGAGCTAACGAAAGCTAAAATGGAGAAAAAAATCACTGGAT
ATACCCACCGTTGATAATCCCAATGGCATCGTAAGAACATTGAGGCATTTCAGTCAGTTGC
TCAATGTAACCTATAACCAGACCGTTCAAGCTGATTAAATGAATCGGCCAACGGGGAGAGGC
GGTTTGC GTATTGGGGCTCTCCGCTTCCCTGCTCACTGACTCGCTCGGTCTGGTCTGGC
TGGGGCGAGGGTATCAGCTCAACTCAAAGGGTAAATACGGTTATCCACAGAAATCAGGGATAAA
CGCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAGGGTAAAAGGCCAGGAACCGTAAAAGGCCGGTG
CTGGCGTTTCCATAGGCTCCGGCCCCCTGACGGAGCATCACAAAAATGGACGCTCAAAGTCAGA
GGTGGCGAAACCGACAGGACTAAAGATAACCAGGGGTTCGGAAAGGCTCCCTCGTGCG
CTCTCCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCGGCTTCTCCCTTCGGGAAGCGTG

Fig. 10D

27/32

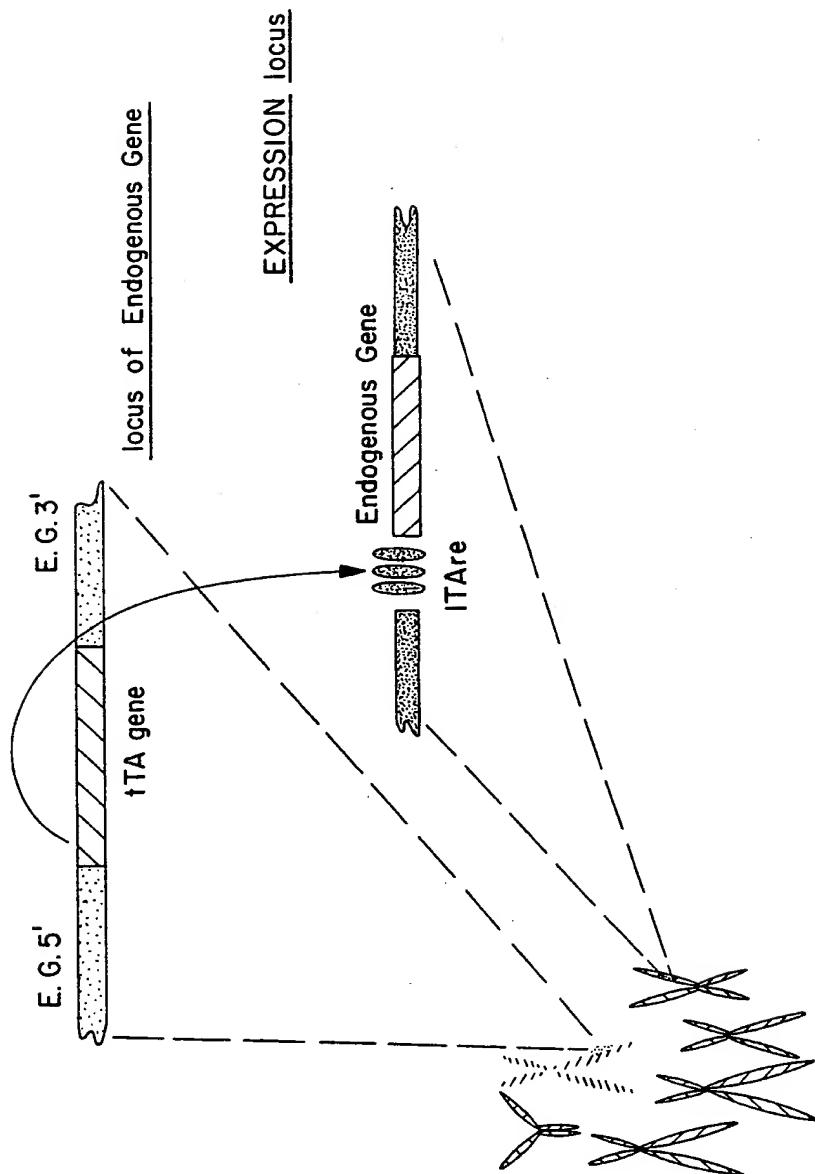
GCGAGGTATGTTAGGGTGTACAGAGTTCTGAAGTGGCTAACCTGGCTAACAGGCTAACACTAGAA
GGACAGTATTGTTAGGGTATCTGGCTCTGGTAAGGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTC
TTGATCCGGCAAACAAACCACCGCTGGTAGCCGGTGGTTTGCAAGCAGATTACCG
CCGAGAAAAAAGGATCTCAAGAAAGATCCTTGATCTTCTACGGGGTCTGACGGCTCAGTGGAA
ACGAAAACCTCACGTTAAGGGATTGGTCATGAGATTATCACAAAGGATCTCACCTAGATCCT
TTTAATTAAAATGAAGTTTAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT
TACCAATGCTTATATCAGTGAGGCACCTATCAGCGATCTGTCTATTTCGTTCATCCATAGTTG
CCTGATCCCCCGTCTGGTAGATAACTACGGATAACGGGGCTTACCATCTGGCCCAGTGGCTGCA
ATGATAACGGCGAGACCCACGGCTCACCGGCTCAGATTATCAGCAATAAACCCAGCCAGCGAA
GGGCCAGCGCAGAAGTGGTCTGCAACTTATCCGCCCTCCATCCAGTCTATTAAATTGTTGCCG

Fig. 10E

GGAAAGCTAGAGTAAGTAGGCCAGTTAACATAGTTGCGCAACGTTGTTGCCATTGCTACAGGC
ATCGTGGTGTCA CGCTCGTCGTTGGTATGGCTTCAGCTCCGGITCCTCCGATCGTTG
GAGTTACATGATCCCCCATGTTGTGCAAAAAGCGGTAGCTCCTCGGTCTCCGATCGTTG
CAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACT
GTCATGCCATCCGTAAGATGCTTTCTGTGACTGGTGAAGTACTCAACCAAGTCATTCTGAGAAT
AGTGTATGGGGGACCGAGTTGCTCTGCCCGCGTCAAATACGGGATAAATACGGCCACATAG
CAGAACCTTAAAGTGTCACTATTGAAAACGTTCTCGGGCGAAAACCTCTCAAGGGATCTTA
CCGCTGTTGAGATCCAGTCGATGTAACCCACTCGTGACCCAACTGATCTCAGCATCTTTA
CTTTCACCCAGGTTCTGGGTGAGCAAAAACAGGAAGGCCAAAATGCCGCAAAAGGGATAAG
GGCGACACGGAAATGTTGAATACTCATACTCTTCCTTCAATATTATTGAAGCATTATCAG
GGTTATGTTCTCATGAGCGGATAACATATTGAATGTTAGAAAAATAACAAATAGGGGTC
CGCGCACATTCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAAAC
CTATAAAAATAGGGGTATCACGGGCCCTTCGTC

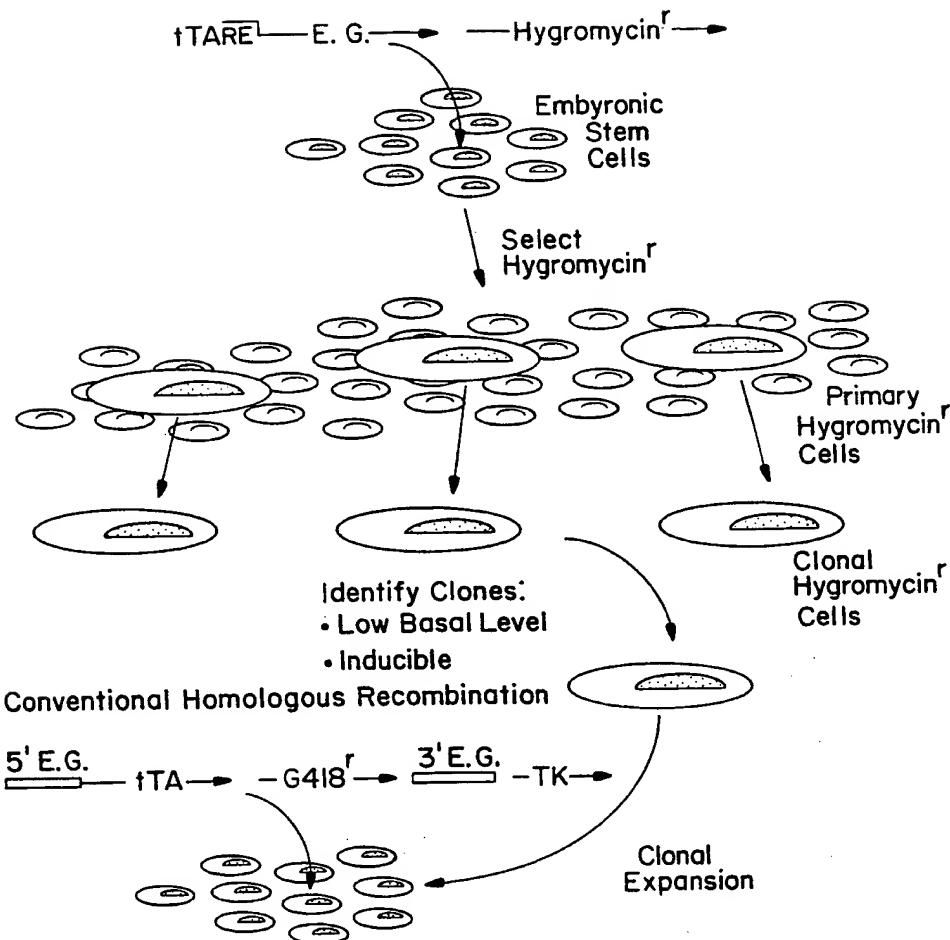
Fig. 10F

FIG. II



SUBSTITUTE SHEET (RULE 26)

FIG. 12



SUBSTITUTE SHEET (RULE 26)

FIG. 13A

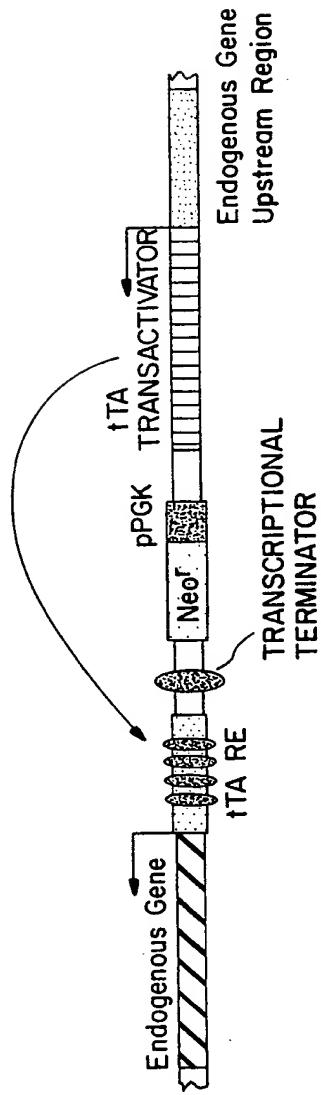


FIG. 13B

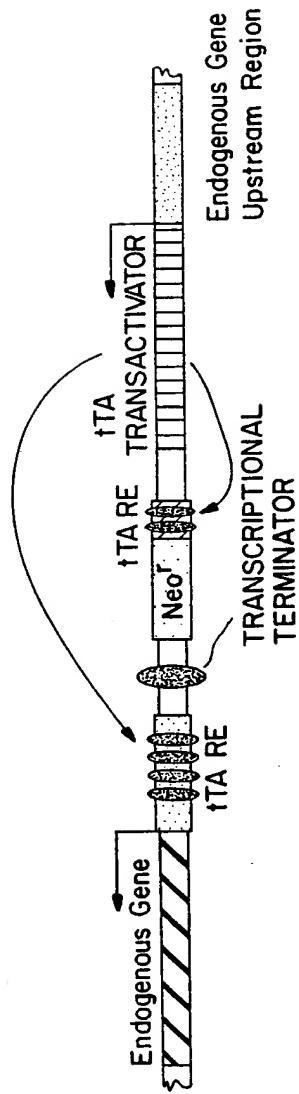
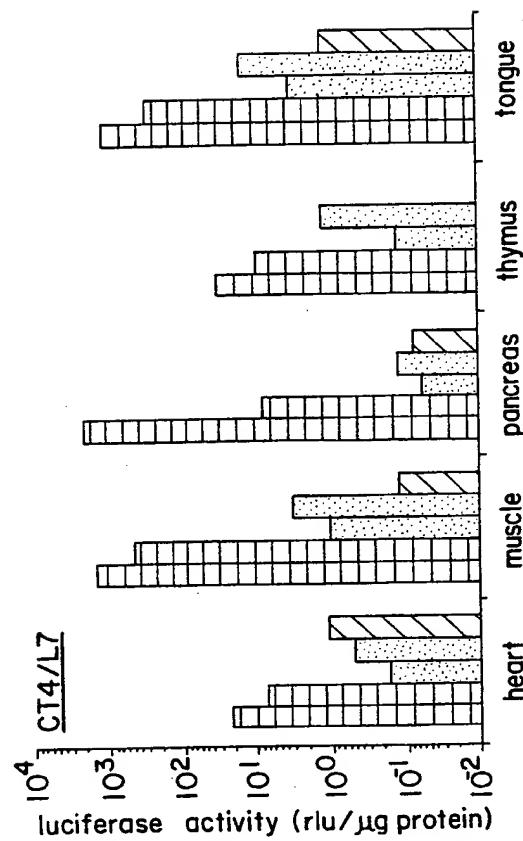


FIG.14



SUBSTITUTE SHEET (RULE 26)

THIS PAGE BLANK (USPTO)